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Latex of *Sciadopitys verticillata* (Thunb.) Siebold and Zuccarini: Antibiotic Properties, Phytochemistry, and Inhibition of Adventitious Rooting of Stem Cuttings.

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ABSTRACT

Latex of *Sciadopitys verticillata* (Thunb.) Siebold and Zuccarini: Antibiotic Properties, Phytochemistry, and Inhibition of Adventitious Rooting of Stem Cuttings

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Sciadopitys verticillata was subjected to 3 propagation treatments designed to inhibit coagulation of its latex-like sap at the cut ends of the stem cuttings. Twenty-four hour soaking in water prior to rooting hormone application significantly enhanced production of adventitious roots. Old wood stem cuttings from shade-grown trees rooted at higher proportions than stem cuttings collected from sun-grown trees. Height, age, and place of origin of the source trees were not important factors in successful rooting. Antibacterial activity against some human pathogens and soil bacterial species was detected in latex application trials but the antibiotic activity was not related to the bacterial Gram reaction. The latex-like sap inhibited none of 4 plant pathogens tested. A suspension of the water insoluble latex-like sap of *S. verticillata* had a pH of 5.8. Antibacterial activity of *S. verticillata* sap was heat stable, which indicates the activity was not protein-based.

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CHAPTER 1

INTRODUCTION

Sciadopitys verticillata (Thunb.) Siebold & Zuccarini, commonly known as the Japanese umbrella pine, is called "Koya-maki" in Japanese and is translated as "Pine of Mount Koya" (Dallimore et al. 1967). The English name "umbrella pine" refers to the whorls of leaves resembling the spokes of an umbrella, as does the Greek root "Sciad", a canopy or umbrella (Figure 1). The species name is derived from the Latin word "verticillus", the whorl of a spindle. *Sciadopitys verticillata* is native to Japan (s. Honshu, Kyushu, and Shikoku Provinces) where it grows as isolated plants and in small isolated patches in humid, high rainfall, mid-elevation cloud forests at 500 - 1000 meters (Yamamoto 1988). The species forms old-growth forests in association with *Chamaecyparis obtusa* (Siebold & Zuccarini) Endlicher (Yamamoto 1988). Old growth forests are forests whose natural cycles of growth have not been disturbed by logging, building roads, or clearing. Seedlings of *S. verticillata* can regenerate beneath the forest canopy, although small gaps with exposed mineral soils constitute preferred establishment sites.

Figure 1 *Sciadopitys verticillata* stem. *Sciadopitys verticillata* has leaves radiating outwards from the stem apex. Most needled conifers have leaves radiating outwards along the entire stem.

Sciadopitys verticillata is an ancient endemic with a highly relictual range. Endemic plants are species are native to a particular region or habitat. In biology, a relict refers to a species that at an earlier time was abundant in a large area but is now found at only one or a few small areas (Stefanovic et al. 1998). The prehistoric range of the genus was far more extensive across both Eurasia and North America with fossils dating to the upper Triassic period 205 - 250 million years ago (Phillipe et al. 2002). Among the oldest of all extant conifers, and already in decline by the time other ancient conifers became widespread in the lower Cretaceous period (100 - 145 million years ago), it can be considered a living fossil (Yamamoto 1988).

Until recently, the taxonomic literature has been discordant on the family classification of the monotypic genus *Sciadopitys* (Schlarbaum and Tsuchiya 1985, Takaso and Tomlinson 1991, Wolff et al. 1996, Axsmith et al. 1998, Stefanovic et al. 1998, Farjon and Ortiz 2003). Monotypic refers to a taxonomic group with only one subgroup at the next lower taxonomic level. For example, a monotypic genus has only 1 specie (Farjon and Ortiz 2003). *Sciadopitys* had previously been assigned to the families Pinaceae and Taxodiaceae, but recent analysis of the genes encoding ribosomal RNA has led to assignment in the family Sciadopityaceae, of which it is the only living member (Stefanovic et al. 1998, Farjon and Ortiz 2003).

The sap of *S. verticillata* is unique among conifers because it is a white, viscous latex-like sap that quickly coagulates when exposed to air (Figure 2). This physical property of the sap may underlie problems in vegetative propagation by rooting stem cuttings (Waxman 1978). Horticulture propagation manuals from the 1970s reported that attempts to root stem cuttings had not been sufficiently successful to justify propagation by cuttings and instead recommended propagating by seed (Waxman 1957, Waxman 1960, Flemer 1961, Waxman 1978, Blazich and Wright 1986, Dirr 1990, Halladin 1991, Goodhart 2000). More recent technical improvements

have increased rooting success. These improved techniques include an overnight soak of cuttings in water to prevent the sap from coagulating and forming a barrier at the cut end, the use of growth regulators and rooting hormones to stimulate meristematic cell division and production of adventitious roots, and inoculation with mycorrhizal fungi as a method for supplying water to the stem cuttings while roots are forming (Halladin 1991, Douds et al. 1995, Taiz and Zeiger 2002). Mycorrhizal (root fungus) fungi form a symbiotic relationship with many plant species in which the fungus delivers water and mineral nutrients to the roots and the plant supplies the fungus with organic products produced by photosynthesis (Douds et al. 1995, Taiz and Zeiger 2002). The highest percent rooting reported to date for *Sciadopitys* were from stem cuttings taken in January and treated with 100 ppm 1-napthaleneacetic acid (NAA) for 24 hours, with rooting taking as long as 8 months (Waxman 1978). A direct correlation between the amount of sap and rooting ability, with low sap correlated to high rooting, led to the supposition that *Sciadopitys verticillata* sap has been presumed to inhibit adventitious root formation (Waxman 1978). High tree-to-tree variability in rooting success has also been reported (Waxman 1978), but the cause of hypothesized tree-to-tree rooting variability is unknown and the elucidation of this type of difference was another goal of the current study.

Figure 2 *Sciadopitys verticillata* latex sap exuding from latcifer ducts. Latex quickly coagulates when exposed to air forming a protective barrier in response to wounding. Stem pictured is approximately 1 cm in diameter. Arrow indicates example of latex sap used in this study.

An adventitious root is a root that arises from a stem rather than from the primary root. The vascular cylinder comprises all the tissues enclosed by the endodermis. It consists of the pericycle and vascular tissues (xylem and phloem). The pericycle, which occurs only in roots, is a single layer of thick-walled, tightly packed cells without intercellular spaces. Lateral roots arise from the pericycle of other roots while adventitious roots are roots that originate directly from the vascular meristems of the shoot. Rhizomes (ground growing stems) and shoots that touch the soil are capable of forming adventitious roots. The adventitious rooting phenomenon is used to propagate plants vegetatively through stem cuttings (Taiz and Zeiger 2002). New roots arise

from meristematic cells. A meristem can retain its embryonic character indefinitely and retains the capacity for cell division and differentiation. Most plants develop a variety of secondary meristems during postembryonic development. Secondary meristems can have a structure similar to that of primary meristems, but some secondary meristems have a quite different structure. Secondary meristems include axillary meristems, inflorescence meristems, floral meristems, intercalary meristems, and lateral meristems. Lateral meristems include the vascular cambium and the cork cambium. Adventitious roots also can be produced from lateral root meristems that develop on stems, as when stem cuttings are rooted to propagate a plant.

The vascular cambium contains 2 types of meristematic cells: the fusiform stem cells and ray stem cells. All meristematic cells may be a potential origin of adventitious roots (Taiz and Zeiger 2002). Two modes of regeneration, direct and indirect, occur in woody plants. The two modes differ in that indirect regeneration involves an intermediate phase (unorganized proliferation of cells) whereas direct regeneration does not (Ye and Droste 1996). Adventitious meristem formation in plants can be induced directly or indirectly (Dandekar et al. 1993).

In plants, unlike animals, cell differentiation is frequently reversible, particularly when differentiated cells are removed from the plant and placed in tissue culture with appropriate nutrients and hormones. Under these conditions, cells de-differentiate (loss of tissue-specific characteristics), and reinitiate cell division. When provided with the appropriate nutrients and hormones these de-differentiated cells can regenerate whole plants. This ability to de-differentiate demonstrates that differentiated plant cells retain all the genetic information required for the development of a complete plant, a property termed totipotency. The exception to this rule is cells that lose their nuclei (Taiz and Zeiger 2002).

Waxman's (1978) propagation study is considered to be the standard by which nursery growers gauge *S. verticillata* stem cutting rooting success. Plant propagators interviewed during the preliminary stages of this study reported that they had never attained Waxman's average rooting percentage (90%) using his or any other propagation techniques. Those interviewed reported their average proportion of stem cuttings producing roots was approximately 5 - 10%, rates that were markedly less than the 90% average reported by Waxman (1978).

In the current study, latex sap from *S. verticillata* was examined to determine if it forms a chemical and/or a mechanical barrier to rooting. Various stem cutting treatments were investigated to determine whether this hypothesized barrier could be overcome by treatments designed to prevent the sap from coagulating at the end of the cutting. Possible seasonal effects were also considered to account for potential differences in sap chemistry during dormant and active growth periods (Table 1).

Table 1 Hypotheses tested in current study

Hypotheses	
Adventitious Rooting	
A	Different aged *S. verticillata* plants used as source for stem cuttings will produce adventitious roots at different proportions.
B	Different individual *S. verticillata* plants used as source for stem cuttings will produce adventitious roots at different proportions.
C	The *S. verticillata* stem cuttings subjected to different treatments will produce adventitious roots at different proportions.
D	The *S. verticillata* stem cuttings collected during different seasonal stages of growth (summer new wood *vs*. winter old wood) will produce adventitious roots at different proportions.
E	The *S. verticillata* stem cuttings collected from trees growing in different light (sun *vs*. shade) environments will produce adventitious roots at different proportions.
Antibiotic Properties	There will be no antimicrobial activity attributable to *S. verticillata* latex.

Sciadopitys verticillata is praised by horticulturists and landscape architects for its disease-resistant and insect-resistant properties. It is well known that plants have evolved an array of biochemical defenses against a range of herbivorous organisms (Francheschi et al. 1998). Sap from *S. verticillata* is generally considered unpalatable to many insects and, with the sap's ability to quickly coagulate, wounds caused by insect damage are quickly sealed (Figure 2). A first line of defense is exudation of resins at the site of attack (Francheschi et al. 1998, Langenheim 2003). A successful defense against insect attack is dependent on the flow rate and sap viscosity, two factors that determine whether insects are pushed out or trapped at the wound site (Langenheim 2003). While factors promoting insect resistance are apparent, factors enhancing the ability to resist disease and infection are unknown. For example, antimicrobial studies of *S. verticillata's* sap have not been reported.

To investigate possible antimicrobial properties of *Sciadopitys* latex, extracts of the latex-like sap was applied to various bacterial cultures. A worldwide increase in premature human deaths caused by antibiotic resistant bacteria has increased the need for new antibiotics (Hubner 2003, Sakagami et al. 2005). Bacterial resistance to antibiotics has significantly increased in recent years along with the need for new antibiotics to combat emerging resistant strains (Hubner 2003, Lin et al. 2005). Hence, a goal of this study was to assess antibacterial properties of *S. verticillata* sap against a panel of diverse human, plant, and soil-dwelling bacterial species (Tables 1 and 2). To assess potential therapeutic benefits to humans, human bacterial pathogens and commensals included in the antibiotic trials were *Escherichia coli*, *Moraxella catarrhalis*, *Neisseria cinerea, Staphylococcus aureus*, and *Staphylococcus epidermidis.* To assess plant protective properties, the plant pathogens *Agrobacterium tumefaciens, Erwinia amylovora, Pseudomonas syringae, and Xanthomonas sp.* were tested. Also included were the soil-inhabiting *Bacillus cereus* and *Bacillus subtilis.*

Studies of latex sap have been conducted to determine components of sap from the Euphorbiaceae (Calvin, 1979, Calvin 1984, Bravo et al. 1994, Liggieri et al. 2004). Latexes of some desert *Euphorbia* species also have been studied as a source of hydrocarbons for petroleum production (Calvin 1979, Calvin 1984). These hydrocarbon analyses have been primarily conducted on latex-producing plants containing a large volatile fraction in the sap. In contrast, sap from *S. verticillata* lacks a volatile fraction (Dorman and Deans 2000, Langenheim 2003). Studies conducted on *S. verticillata* have isolated biflavonoids from leaves (Wollenweber et al. 1998). Because few studies have examined properties of *S. verticillata's* latex sap, a goal of this study was to determine the basic chemical and physical properties of the sap that may be relevant to vegetative propagation and antimicrobial activity. Analysis of the physical characteristics and

chemical properties could also be useful in future studies on sap coagulation and the breakdown of sap to a form that does not interfere with meristematic cell development.

Analysis of pH of *Sciadopitys* sap was conducted to determine if the cause of bacterial inhibition could be due to the environment being incompatible with microbial growth when the sap was applied. Environmental pH has a major effect on the growth of microorganisms with each organism having a well-defined pH optimum for growth (Madigan and Martinko 2006). Most organisms show a growth range of 2 - 3 pH units (Madigan and Martinko 2006). Most natural environments have a pH value between 5 and 9 and organisms with optima in this range are most common (Madigan and Martinko 2006). The bacteria used in this study prefer an environment in the pH range of 5.0 - 9.0 (Madigan and Martinko 2006).

CHAPTER 2

METHODS

Study Organism

Sciadopitys verticillata

Sciadopitys verticillata is an evergreen tree growing to 20 - 30 meters tall and up to 1 meter in trunk diameter at breast height, with dense, heavy branching with luxuriant foliage. Individuals can be either single- or multi-stemmed (Figure 3). Bark is thick, soft, red-brown, and stringy. Leaves are of two types: scale leaves are on the stem, brown, 1 - 3 mm, widely spaced between the nodes on long shoots, and clustered in a tight spiral pseudowhorl subtending the typical photosynthetic leaves located at the apex of both long and short shoots (Dallimore et al. 1967).

Figure 3 Mature *Sciadopitys verticillata*. Mature *Sciadopitys verticillata* growing on the James H. Quillen Veterans Administration grounds at Mountain Home, Tennessee. This 10-meter-tall tree was the source of sap for the antibiotic trials of this study and was 1 of the sources of stem cuttings for the propagation trials

Plant specimens used in this propagation and antibiotic study were growing in Carter, Unicoi, and Washington Counties of northeastern Tennessee. Six trees were used as source material for stem cuttings in this study. These six plants differed in growing environment, age, and propagation source. Some plants from each age group were growing in a sunny location and some were growing in a shady location. The age groups, which are correlated with heights, were: Group 1; less than 1.5 meters tall (less than 10 years old); Group 2, between 1.5 to 3 meters tall (10 to 20 years old); and Group 3, larger than 3 meters tall (older than 20 years old).

Tree ID #1. V.A. Tree - A reproductively mature tree 10 meters tall at the James H. Quillen V.A. Medical Center campus, Mountain Home, TN growing in a sunny location. This specimen was planted in the 1940s by Colonel Harr and his wife, who brought the tree from Japan (Figure 3).

Tree ID #2. F.L. Tree - A 15-year-old tree 3 meters tall located in a sunny location at Dr. Foster Levy's home in Unicoi, TN. This tree was purchased from Happy Valley Tree Farm in Elizabethton, Tennessee. Source of plant was from a cutting taken from a 2 meters tall container-grown plant purchased from Rogers Associates in Canby, Oregon. Two trees used in this study were genetically identical (# 2 and #4) and growing in similar sun environments. While genetics may also play a role in successful rooting, this factor was not considered in the current study.

Tree ID #3. H.C. Tree - A 3 meters tall plant that was purchased in 1989 from Kinsey Gardens in Knoxville. The source of the original plant is unknown; however, it was a grafted tree. The plant is growing in a sunny dry location approximately 2 miles north of E.T.S.U. in Washington County, TN.

Tree ID #4. L.N. Tree - A 1 meter tall plant, growing at Laurels Nursery, in Carter County, TN, that was rooted in 1990 from a branch that was broken on a 6' tall container grown

plant purchased from Rogers Associates in Canby, Oregon. This plant is growing in a dry sunny location at 2,817 feet above sea level.

Tree ID #5 G.M. Tree - A 30-year-old tree 8 meters tall located in a shady location approximately 2 kilometers west of E.T.S.U. in Washington County, TN (Figure 4). The tree was purchased from a mail order company approximately 30 years ago. The owner believes that the tree was shipped out of New Jersey. Cuttings from this plant were difficult to stick in media because the stems were thin and flexible. Thus, a hole had to be made in the media with a pencil before cuttings could be stuck.

Figure 4 *Sciadopitys verticillata* growing in a shady environment. Guy Mauldin's 30-year-old 8-meters-tall *Sciadopitys verticillata* located in a shady location approximately 1 mile west of E.T.S.U. This is Tree ID #5 and was used as a source for stem cuttings.

Tree ID #6. M.M. Tree – A 2 meters tall plant that was purchased from Evergreen Nursery in Johnson City, TN in 2002 and is growing in Cater County, TN. This plant was

originally acquired by Evergreen Nursery and shipped from Oregon to Johnson City, TN, for resale.

The six trees used in this study were assigned identification numbers and an abbreviated alphabetical identifier for data collection purposes. The alphabetical identifier uses the first letters of the owners' names (Table 2).

Table 2 Source of stem cuttings. Table indicates tree identification number, source of stem cuttings, growing environment, approximate tree age and height, and provenance for trees used in propagation trials.

Tree ID #	Source	Environment	Approx. Age and Height	Provenance
#1 (V.A.)	V.A. Campus, corner of Maple and Dogwood streets	Sunny	60-year-old, 10-meters-tall tree	Believed planted in the 1940's by a Colonel Harr and his wife, who brought the tree from Japan.
#2 (F.L.)	Dr. Foster Levy's home in Unicoi County, Tennessee.	Sunny	15-year-old, 3-meters-tall tree	Purchased from Happy Valley Tree Farm in Elizabethton, Tennessee.
#3 (H.C.)	Hugh Conlon residence, Washington County, Tennessee.	Sunny	3-meters-tall tree	Purchased in 1989 from Kinsey Gardens in Knoxville. The source of the original plant is unknown, however it is a grafted tree.
#4 (L.N.)	Laurels Nursery, in Carter County, Tennessee	Dry sunny location	1-meter-tall tree	Rooted in 1990 from a branch that was broken on a 6' tall container grown plant purchased from Rogers Associates in Canby, Oregon.
#5 (G.M.)	Guy Mauldin's residence, Washington County, Tennessee.	Shady	30-years-old, 8-meters-tall tree.	Purchased from a mail order company approximately 30 years ago. The owner believes that the tree was shipped out of New Jersey.
#6 (M.M.)	Terry "Mud" Montgomery's residence, Carter County, Tennessee.	Shady	2-meters-tall tree	Purchased from Evergreen Nursery in Johnson City, Tennessee in 2002.

Bacteria

The bacterial strains selected for this study occupy various ecological niches and included human commensals/pathogens, soil dwelling species, and plant commensals/pathogens (Figure 5). The microbial samples in the trials included both Gram-positive and Gram-negative bacteria (Table 3). Commensal bacteria live in association with a host but they do not adversely affect the host. Bacteria are considered pathogens if they harm the host. Sources of bacteria were laboratory strains from East Tennessee State University, University of Tennessee, Knoxville, and the James H. Quillen Veterans Administration Department of Infectious Diseases, located in Mountain Home, Tennessee (Table 3, Figure 5).

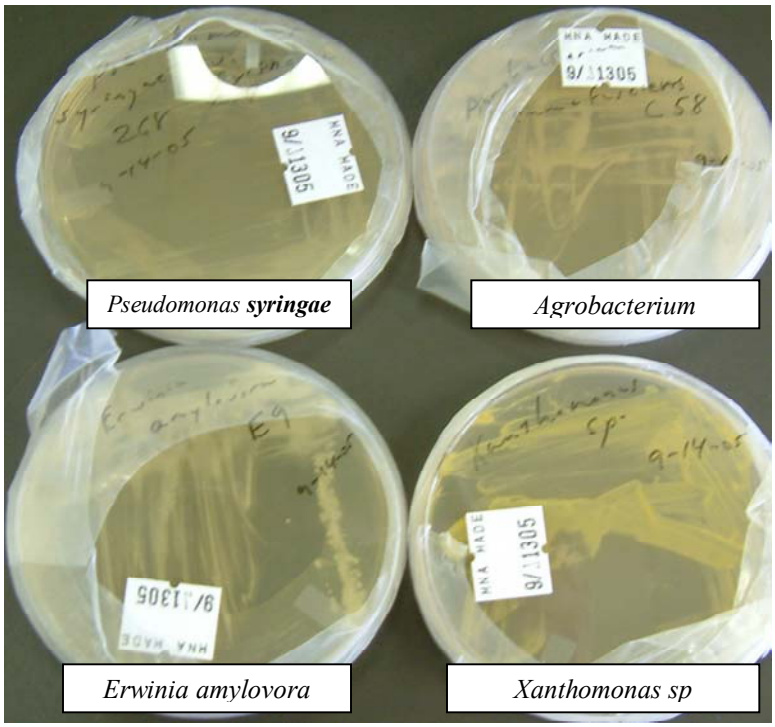


Figure 5 Plant pathogens used in antimicrobial trials. Four plant pathogens tested in this study were bacterial strains provided by the University of Tennessee, Knoxville, TN. Bacteria strains were retained for future studies or to verify results.

Table 3 Bacteria used in this study. Species, strain, Gram reaction (+ or -), source, and characteristics of bacteria used in this study.

Species and Strain	Gram + or -	Source	Characteristics/Remarks
Staphylococcus aureus NCTC 8325	+	James H. Quillen Veterans (V.A.) Administration Department of Infectious Diseases	*S. aureus* is a spherical bacterium (coccus) that appears in pairs, short chains, or bunched, grape-like clusters. Some strains are capable of producing a highly heat-stable protein toxin that causes illness in humans.
Staphylococcus epidermidis VA5A1	+	(V.A.) Department of Infectious Diseases	Human commensal common on skin.
Moraxella catarrhalis ATCC 252328	-	(V.A.) Department of Infectious Diseases	Human commensal/pathogen of respiratory tract
Neisseria cinerea ATCC 14685	-	(V.A.) Department of Infectious Diseases	Human commensal/pathogen of respiratory tract
Escherichia coli DH5a	-	(V.A.) Department of Infectious Diseases	Human commensal common in intestinal tract.
Bacillus cereus WM20-1	+	East Tennessee State University (E.T.S.U.) Department of Biological Sciences.	Common soil microbe
Bacillus subtilis 168TF	+	(E.T.S.U.) Department of Biological Sciences.	Common soil microbe
Pseudomonas syringae	-	University of Tennessee	Pathogenic to lilac
Agrobacterium tumefaciens	-	University of Tennessee	Causes crown gall tumor on alfalfa
Xanthomonas sp.	-	University of Tennessee	Associated with infections in humans, causes bacteraemia. Causes bacterial leaf spot on peppers and tomato and black rot on cabbage.
Erwinia amylovora	-	University of Tennessee	Bacteriophage host

Propagation Trials

Stem cuttings were taken in the mornings of overcast days. Harvesting the stems while overcast prevented the cuttings from drying out, which could affect the success of the rooting. Each stem cutting included one whorl of photosynthetic leaves with an associated apical bud. All cuttings were scored laterally approximately 3 centimeters from the apical end towards the basal end of the cutting just before treatment (Table 4, Figure 6). Stem cuttings averaged 15 centimeters in length and had approximate average diameters of 0.5 centimeters.

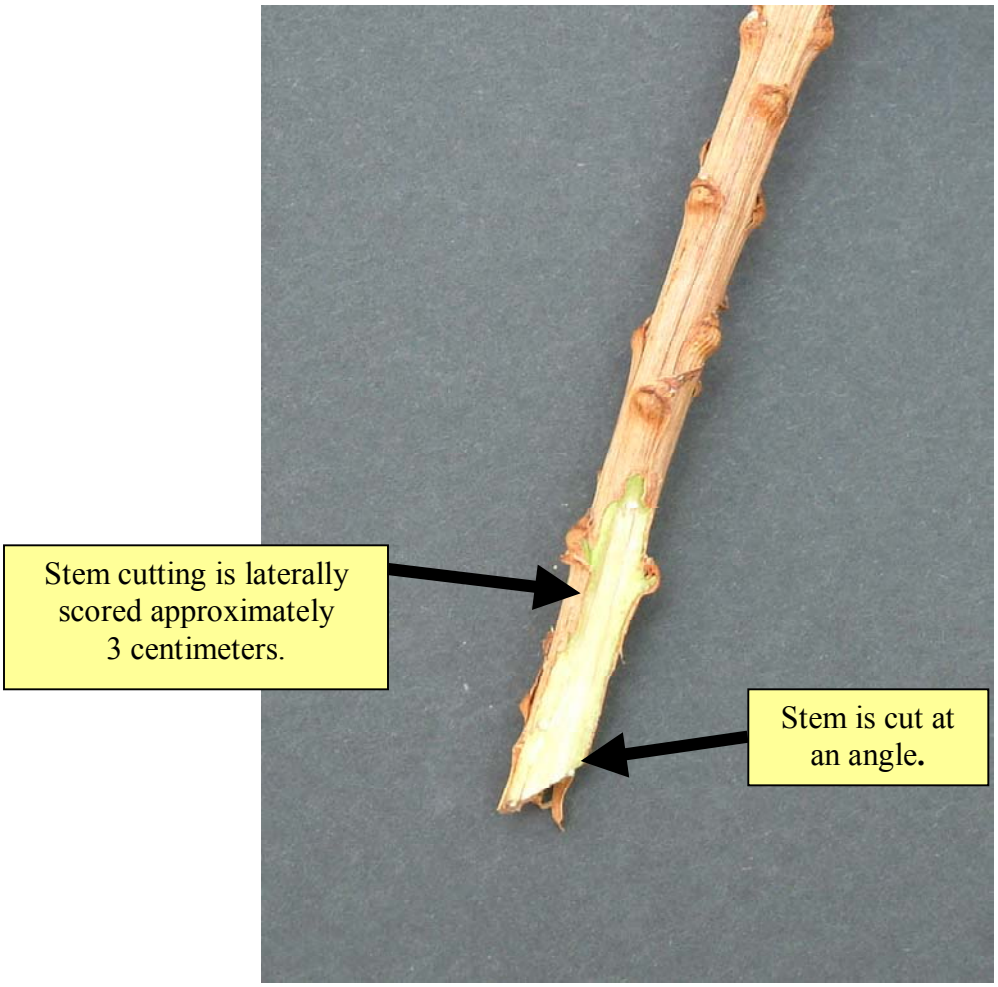


Figure 6 Stem cutting prepared for propagation treatment. Stem is cut at an angle and laterally scored approximately 3 centimeters before applying hormones or soaking treatment. They had 1 to 2 whorls of photosynthetic leaves in addition to any apical buds.

Table 4 Propagation treatments**.** Four propagation treatments were used in this study.

Treatment	Description
Control	Fresh basal cut, 1 minute in Dip-n-Grow rooting hormone (Figure 7) at 1:5 dilution.
24 hour water soak	Fresh basal cut, soaked in water 24 hours, followed by fresh basal cut, 1 minute in Dip-n-Grow rooting hormone (Figure 7) at 1:5 dilution (Figure 8).
Cut under water/24 hour water soak	Cuttings were given a fresh basal cut under water to exclude exposure to air and soaked in water for 24 hours. Fresh basal cuts were then made again and cuttings were treated for 1 minute in Dip'N-Grow at 1:5 dilution.
24 hour detergent/water soak	Cuttings were given a fresh basal cut and soaked in 100 ml of water with 0.5 ml of Tween-20 detergent for 24 hours. Fresh basal cuts were then made and cuttings were treated for 1 minute in Dip'N-Grow in 1:5 dilution.

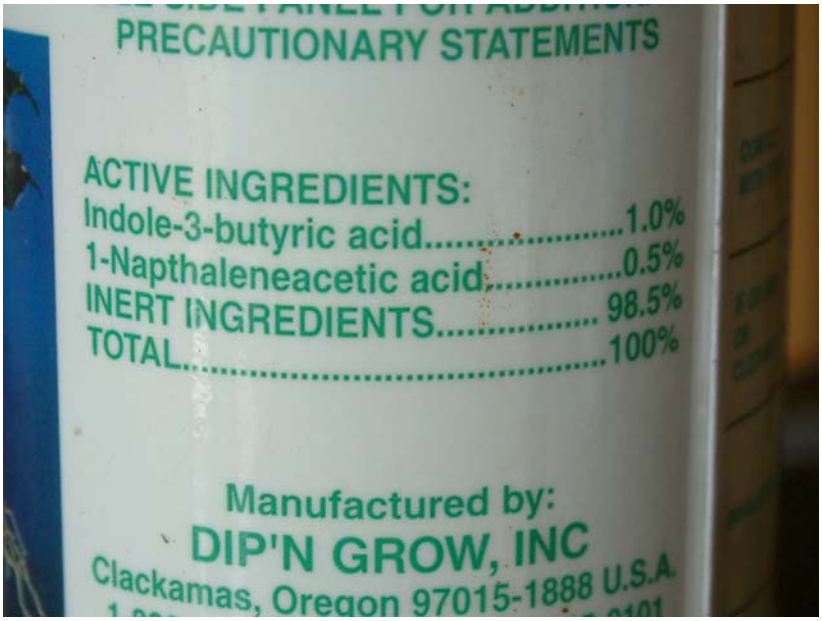


Figure 7 Rooting hormone used in propagation trials. Rooting hormone was manufactured by Dip'N Grow, Incorporated of Clackamas, Oregon. Manufacturer's recommended concentration for "difficult to root" species was used in propagation trials.

Figure 8 *Sciadopitys verticillata* stem cuttings soaking. *Sciadopitys verticillata* stem cuttings were subjected to various overnight soaking treatments.

Following treatments listed in Table 4 all cuttings were immediately placed to a depth of approximately 7 centimeters in a soil-less media composed of 50% peat moss and 50% perilite in tree trays with cells 10 centimeters deep and 5 centimeters square and placed under intermittent mist (Figure 9). This media ratio allowed for good drainage yet maintained an adequate level of moisture near the cut end, the area of potential adventious root formation. The relatively high volume of perilite in the medium provided for quick water drainage, which helped prevent dampening off, a form of fungal rot. No fungicides were used.

The cuttings were placed in an enclosed greenhouse at East Tennessee State University, with year-round 50% shade cloth above the cuttings (Figure 9). The shade cloth was needed to control the rate of transpiration and prevent sunburn of cuttings during the experiment. The temperature was maintained at 23 - 30 C.

Figure 9 *Sciadopitys verticillata* cuttings in greenhouse. *Sciadopitys verticillata* stem cuttings were incubated under intermittent mist in a climate-controlled greenhouse located on the campus of East Tennessee State University.

Approximately 90 - 100% humidity was maintained around the cuttings. High humidity in the air sustains the water balance in the plant cells by reducing transpiration. An intermittent mist regime of "10 seconds on and 3 minutes off" was used during daylight hours to maintain humidity levels (Figure 9).

Randomly selected stem cuttings were visually inspected monthly for evidence of root formation and for new shoot growth (Figure 10). Shoot growth data were collected as an initial indicator of the viability of the cuttings until all shoot growth stopped (3 months) after which shoot growth data were no longer collected. Rooting success was assessed 6 months after planting. Roots were gently separated from the media by hand and visually inspected to categorize rooting success or failure. The following five categories were used to classify rooting activity: Heavily Rooted, Lightly Rooted, Callused, Viable, and Dead (Figures 10, 11, and 12).

Stem cuttings with more than 5 roots longer than 3 centimeters were categorized as “Heavily Rooted” (Figures 10 and 11). Stem cuttings with 5 or fewer roots, all shorter than 3 centimeters were scored as “Lightly Rooted” (Figure 10). Stem cuttings that had formed a callus, but had not differentiated roots, were scored as “Callused” (Figure 10). Stem cuttings that maintained green needles but had not rooted or callused were scored as ”Viable”. Stem cuttings that had dropped their needles, turned brown, and/or rotted were scored as “Dead” (Figure 12).

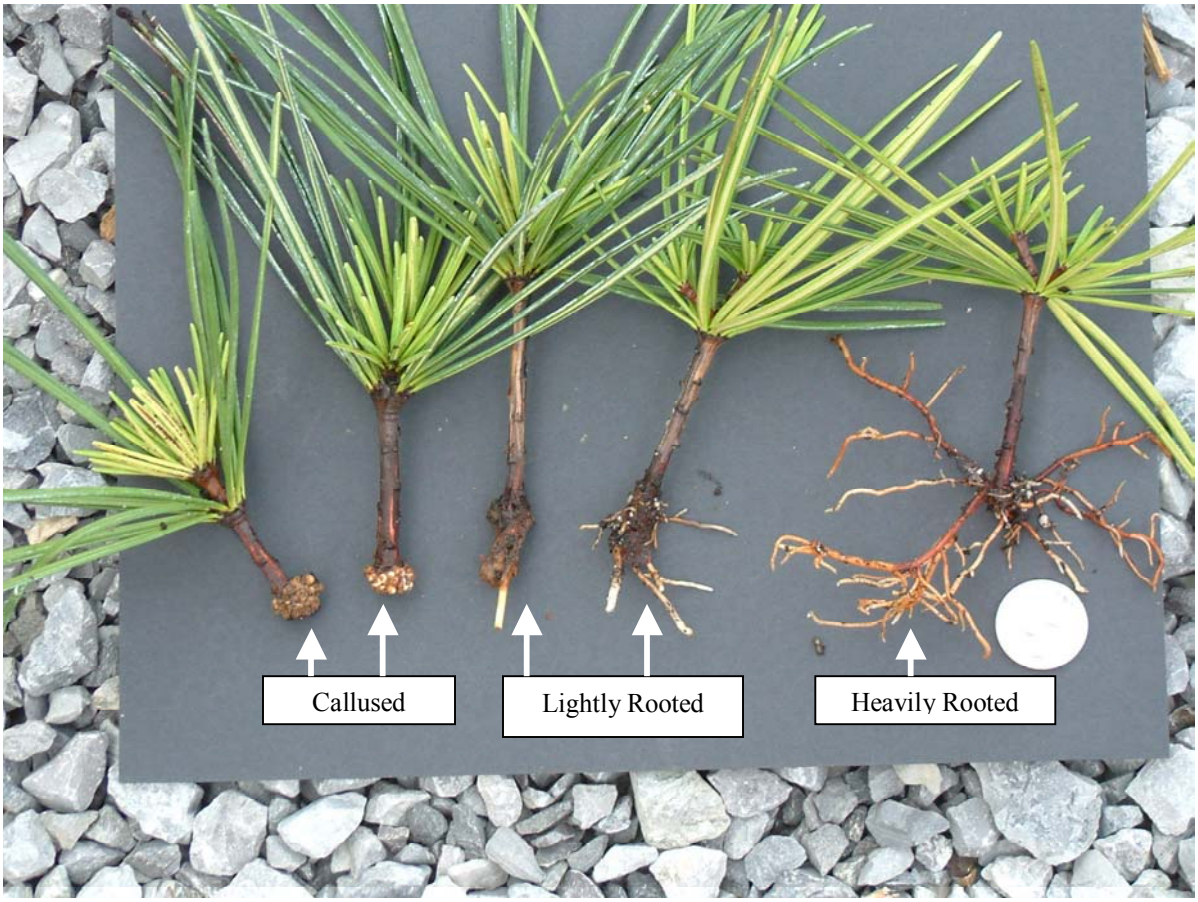


Figure 10 Callused and rooted stem cuttings. *Sciadopitys verticillata* stem cuttings callused, lightly rooted, and heavily rooted. Heavily rooted stem cutting is beside a U.S. quarter as a size reference. The 2 callused stem cuttings were from trees growing in the sun.

Figure 11 Heavily rooted *Sciadopitys verticillata* stem cutting. Close view of a typical heavily rooted *Sciadopitys verticillata* stem cutting produced in the propagation trials of this study. Stem cuttings were classified as heavily rooted if they had produced several roots longer than 3 centimeters.

Figure 12 Dead *Sciadopitys verticillata* stem cuttings. Stem cuttings were classified as dead when leaves turned brown and dropped as in this tray of *Sciadopitys verticillata* stem cuttings subjected to alcohol and water treatment.

Categorical data were used in the data analysis because propagation of stem cuttings can yield 1 of 3 functionally relevant results; stem cuttings can produce adventitious roots, they can produce callus, or they die. The actual length or number of roots was not considered to be as biologically informative in this study relative to the frequencies of rooting success or failure, but heavy and light rooting categories were included to distinguish relative root quality and quantity. In regard to the goal of enhancing rooting success, whether a cutting has 1 root or 5 roots was less important than whether the stem cuttings did or did not produce adventitious roots.

Comparisons of frequencies were conducted using the chi square test for heterogeneity of frequencies. P-values of $\leq$ 5% were considered significant.

Antibiotic Trials

Antibiotic trials were conducted in laboratories at East Tennessee State University, Johnson City, Tennessee and at the James H. Quillen Veterans Administration Laboratory of Infectious Diseases, in Mountain Home, Tennessee. Two laboratories were used due to convenience, scheduling conflicts, and to prevent possible cross-contamination of other ongoing experiments. Similar laboratory equipment and supplies were available at both locations. Working solutions of chemicals used in this study were made for transportation between locations to ensure consistency in the mixtures used in this study.

Sap for antibiotic trials was collected from the V.A. and L.N. trees, (Table 2, Figure 3). Fresh stem cuttings were collected by removing several branches, each approximately 20 cm in length, by cutting branches with sterile pruners. Cuttings were taken to the laboratory for the sap extraction procedure (Figure 13).

Plant Sap Extracts

To surface sterilize plant stems, cuttings were dipped in a 10% bleach solution for 15 seconds, rinsed 4 times with sterile distilled water, patted dry with a sterile towel, and then allowed to air dry approximately 3-5 minutes. Fresh cuts were then made to the stem with a sterile scalpel, so that uncontaminated sap could be collected into sterile microfuge tubes containing 600 microliters (µl) of sterile 0.15 M NaCl.

Test Plates

Frozen stocks of each bacterial species were spread on petri dishes (plates) containing Tryptic Soy agar (TSA) and incubated overnight at 37° C. Individual colonies of each strain of bacteria were mixed with 2 ml of 0.15 M NaCl in a test-tube to a density of 0.5 McFarland Equivalence Turbidity Visual Standard to produce a standard concentration of approximately 1 x 10^8 bacterial cells per milliliter. For antibiotic activity assays, a lawn of bacteria was spread on Mueller Hinton agar media plates by dipping a sterile cotton swab into the NaCl/bacteria suspension, removing the excess by lightly rolling the swab against the side of the tube, and streaking the plates 3 times at progressive 60° angles. Petri dishes were sectored into test areas, labeled with the date, the bacteria strain, and the treatment. To allow bacteria to absorb prior to sap applications, plates were incubated 15-30 minutes at 37° C prior to applying sap extracts. Incubation at 37° C was conducted to allow time for bacteria to become established in the growth media and thus avoid bacteria being displaced by the sap applications.

SapTreatments

Each treatment used 5 µl of sap solution gently pipeted onto bacterial lawns. There were three replicates of each treatment.

A. Solvent control: To verify that the solvent itself was not causing inhibition of bacteria growth, 0.15 M NaCl was used as a control on each plate.

B. Bleach control: In a second control, a wooden pencil that was subjected to the sterilization procedure was directly touched to media. This trial was designed to verify that the sterilization procedure used on the stem cuttings was not causing antibiotic activity.

C. Room temperature sap treatment: Room temperature sap treatments consisted of sap collection in sterile micro-centrifuge tubes by making a fresh sterile cut at the basal end of cuttings, then "milking" approximately 50 µl of the sap into 600 µl of a 0.15 M NaCl solution using hemostats. Following a 5-second vortex, 3 drops, each 5 µl, were placed on plates. The room temperature sap mixture was used as the source of sap for the remaining antibacterial assays.

D. Heat treatment at 65 - 80 C: In a heat treatment, the sap mixture was heated to 65 - 80 C for 10 minutes in a boiling water bath, and then cooled prior to application.

E. Heat treatment 100 C: In another heat treatment, the sap mixture was heated to 100 C in a boiling water bath, then cooled prior to application.

F. Latex treatment: The room temperature mixture was spun for 45 seconds at 14,000 revolutions per minute in a microfuge, the supernatant was discarded

and the resultant white pellet was re-suspended in 50 µl 0.15 NaCl. This procedure yielded a 12x concentration of the insoluble latex fraction.

G. Supernatant Treatment: Supernatant treatment used the supernatant from treatment 'F'.

H. Dilution treatment (100:1): 5 µl of the latex pellet from treatment 'F' was suspended in 500 µl of 0.15 M NaCl followed by vortexing for approximately 15 seconds before applying drops to test plates.

Petri dishes containing bacteria were incubated overnight at 37 C and then were visually inspected for bacterial inhibition zones. An inhibition zone indicated that the bacteria did not grow where the treatment was applied.

Data collected from the antibiotic trials was categorical. Categorical data does not include quantitative measurements but places each data point into a category. The presence of a circular area where bacteria did not grow on the plates indicated growth inhibition caused by the treatment. Because the question posed by this study, (does the sap have any antibiotic properties?), can be answered by either a yes or a no, there was no need to quantify the extent of inhibition zones.

Chemical Properties

Chemical and physical analysis of the sap from *Sciadopitys verticillata* was performed at East Tennessee State University. Sap for chemical and physical analysis was collected from the Laurels Nursery tree, ID #4 (Table 2). Fresh stem cuttings were collected by removing several branches, each approximately 20 cm in length, by cutting branches with sterile pruners. Cuttings

were placed in a clean plastic bag and transported to the laboratory for the sap extraction procedure (Figure 13).

Figure 13 Latex sap extraction. Latex was extracted or "milked" into saline solution or distilled water for antibiotic trials and chemical studies.

Latex sap used for pH determination was collected in sterile micro-centrifuge tubes by making a fresh sterile cut, then "milking" the sap out with hemostats (Figure 14). Approximately 50 µl of sap was suspended in 5 ml distilled water by vortexing for 30 seconds. The pH of the sap suspension was determined using an electronic pH meter.

For sap solubility, 2 solvents were tested; water (H_2O) and 90% ethanol (C_2H_6O). Solubility of *S. verticillata* latex sap in ethanol and water were compared using a spectrophotometer, which measures light transmittance through a solution. Latex sap used in solubility analysis was collected in sterile micro-centrifuge tubes by making a fresh sterile cut, then "milking" the sap out with hemostats (Figure 13). The largest peak registered by the spectrophotometer was at approximately 420 nanometers (nm) and was chosen to be the wavelength used for solubility comparisons. The optical density (OD) of each solvent was analyzed at 420 nm to determine a baseline from which comparisons could be made.

Approximately 3 µl of *S. verticillata* sap was suspended in 2,700 µl of each solvent by vortexing for approximately 30 seconds. Sap suspensions were immediately analyzed for optical density at 420 nm. Optical density was used to determine turbidity of the sap suspensions. Sap suspensions were then centrifuged for 2 minutes to separate the sap from the solvent. Supernatant was removed using a sterile pipette and the optical density at 420 nm determined.

CHAPTER 3

RESULTS

Propagation Trials

Obvious phenotypic differences between stem cuttings were observed when cuttings were placed next to each other. Stem cuttings collected from source trees growing in the sun were longer and more robust, less flexible, woodier, and larger in diameter than cuttings from source trees in the shade (Figure 14). To a lesser extent, similar phenotypic differences were observed within a tree where 1 side of the tree was shaded and the other side was in the sun. The cuttings from shaded source trees were difficult to score prior to treatments and they were too flexible to directly stick in soil media. Hence, a pencil was used to punch a hole in the soil to enable sticking of the more flexible stem cuttings.

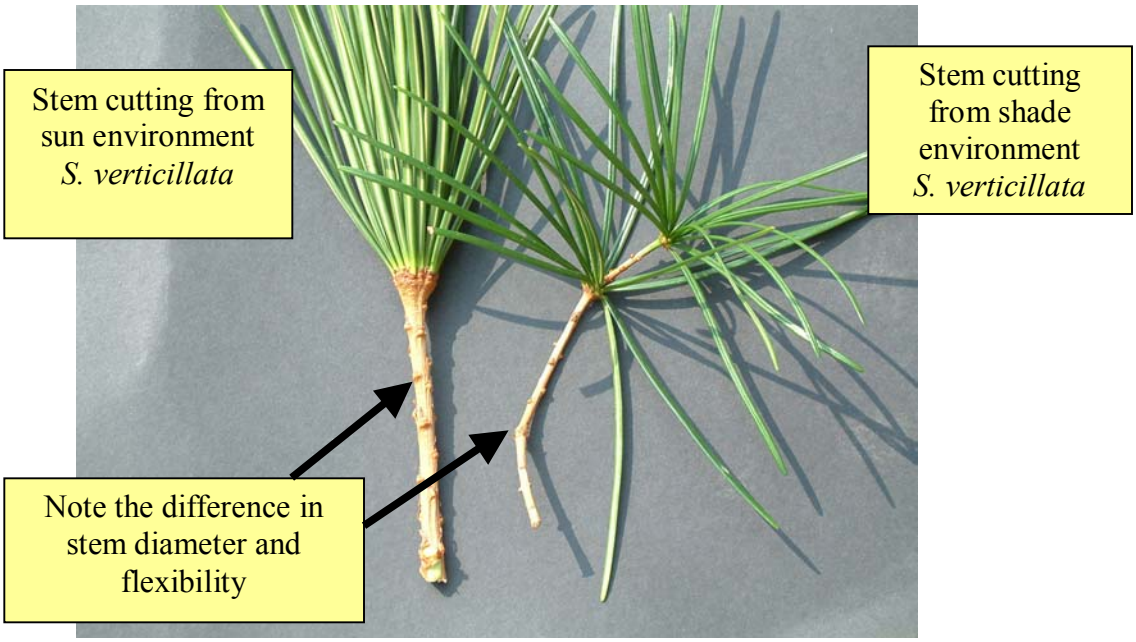


Figure 14 Phenotypic differences in stem cuttings. Phenotypic differences between cuttings collected from trees growing in the sun and shade. Cuttings collected from trees growing in the sun were less flexible and larger in diameter than cuttings collected from trees growing in the shade. To a lesser extent, the phenotypic differences were also observed on the same tree where 1 side of the tree was shaded and the other side was in the sun.

Because nursery growers often use isopropyl alcohol to aid in the removal of conifer sap from tree-shearing tools, preliminary propagation trials included an aqueous isopropyl alcohol soaking treatment. In this treatment, 100 ml isopropyl alcohol (85%) was mixed with 100 ml distilled water (final concentration = 42.5%), then the cut end of stem cuttings were submerged for 24 hours. All cuttings subjected to this treatment turned brown, dropped leaves, and died within 1 week (Figure 12). Therefore, isopropyl alcohol treatments were discontinued.

Comparisons were made between the rooting ability of the cuttings from various tree sources, between the different light environments (sun *vs*. shade) of source trees, between cuttings taken during different seasons (old wood or new wood), and between the different treatment techniques used in this study. Microsoft Excel computer software was used for calculations, data analysis, and graphic representations. The number of cuttings subjected to each trial with proportions of stem cuttings dying, producing callus, and rooting are indicated for the old wood and the new wood in Tables 5 and 6.

Table 5 March (old wood) propagation trials. Spreadsheet with data from the March old wood propagation trials.

Source ID (environment and size)	Treatment	# of Cuttings Stuck	Dead	%	Viable	%	Callus	%	Lightly Rooted	%	Heavily Rooted	%	Percent Rooted
VA	Control	30	2	6.7	7	23.3	18	60	2	6.7	1	3.3	10
(Sunny, Tall Ht.)	24 Hr.H2O Soak	30	1	3.3	4	13.3	18	60	4	13.3	3	10	23.3
	Cut under H2O	30	0	0	4	13.3	17	56.7	6	20	3	10	30
	Detergent	30	3	10	10	33.3	13	43.3	3	10	1	3.3	13.3
FL	Control	10	0	0	1	10	7	70	1	10	1	10	20
(Sunny, Med. Ht.)	24 Hr.H2O Soak	10	0	0	1	10	5	50	2	20	2	20	40
	Cut under H2O	10	1	10	2	20	2	20	3	30	2	20	50
	Detergent	10	3	30	4	40	2	20	1	10	0	0	10
HC	Control	10	1	10	3	30	5	50	1	10	0	0	10
(Sunny, Med. Ht.)	24 Hr.H2O Soak	10	1	10	0	0	5	50	3	30	1	10	40
	Cut under H2O	10	0	0	0	0	6	60	3	30	1	10	40
	Detergent	10	2	20	3	30	3	30	2	20	0	0	20
LN	Control	10	2	20	3	30	4	40	1	10	0	0	10
(Sunny, Sm. Ht.)	24 Hr.H2O Soak	10	0	0	1	10	5	50	2	20	2	20	40
	Cut under H2O	10	0	0	1	10	4	40	3	30	2	20	50
	Detergent H2O	10	3	30	4	40	2	20	1	10	0	0	10
GM	Control	25	7	28	5	20	2	8	6	24	5	20	44
(Shady, Tall. Ht.)	24 Hr.H2O Soak	25	7	28	1	4	1	4	7	28	9	36	64
	Cut under H2O	25	8	32	1	4	0	0	7	28	9	36	64
	Detergent	25	10	40	6	24	1	4	4	16	4	16	32
MM	Control	15	6	40	2	13.3	2	13.3	3	20	2	13.3	33.3
(Shady, Sm. Ht.)	24 Hr.H2O Soak	15	5	33.3	1	6.7	1	6.7	4	26.7	4	26.7	53.3
	Cut under H2O	15	4	26.7	0	0	0	0	6	40	5	33.3	73.3
	Detergent	15	7	46.7	1	6.7	2	13.3	5	33.3	0	0	33.3

Table 6 June (new wood) propagation trials. Spreadsheet with data from the June new wood propagation trials.

Source ID (environment and size)	Treatment	# of Cuttings Stuck	Dead	%	Viable	%	Callus	%	Lightly Rooted	%	Heavily Rooted	%	Percent Rooted
VA	Control	30	2	6.7	7	23.3	18	60	2	6.7	1	3.3	10
(Sunny, Tall Ht.)	24 Hr.H2O Soak	30	1	3.3	10	33.3	15	50	2	6.7	2	6.7	13.3
	Cut under H2O	30	3	10	5	16.7	15	50	4	13.3	3	10	23.3
	Detergent	30	4	13.3	11	36.7	12	40	2	6.7	1	3.3	10
FL	Control	10	2	20	1	10	5	50	1	10	1	10	20
(Sunny, Med. Ht.)	24 Hr.H2O Soak	10	1	10	2	20	3	30	2	20	2	20	40
	Cut under H2O	10	2	20	1	10	2	20	4	40	1	10	50
	Detergent	10	4	40	2	20	2	20	1	10	1	10	20
HC	Control	10	1	10	3	30	5	50	1	10	0	0	10
(Sunny, Med. Ht.)	24 Hr.H2O Soak	10	2	20	0	0	6	60	2	20	0	0	20
	Cut under H2O	10	2	20	0	0	5	50	3	30	0	0	30
	Detergent	10	4	40	3	30	2	20	1	10	0	0	10
LN	Control	10	4	40	3	30	2	20	1	10	0	0	10
(Sunny, Sm. Ht.)	24 Hr.H2O Soak	10	1	10	2	20	4	40	2	20	1	10	30
	Cut under H2O	10	2	20	2	20	2	20	3	30	1	10	40
	Detergent	10	4	40	4	40	1	10	1	10	0	0	10
GM	Control	25	12	48	7	28	1	4	3	12	2	8	20
(Shady, Tall. Ht.)	24 Hr.H2O Soak	25	9	36	6	24	1	4	6	24	3	12	36
	Cut under H2O	25	9	36	5	20	0	0	7	28	4	16	44
	Detergent	25	11	44	8	32	1	4	3	12	2	8	20
MM	Control	15	7	46.7	5	33.3	2	13.3	1	6.7	0	0	6.7
(Shady, Sm. Ht.)	24 Hr.H2O Soak	15	6	40	4	26.7	1	6.7	3	20	1	6.7	26.7
	Cut under H2O	15	6	40	4	26.7	0	0	4	26.7	1	6.7	33.3
	Detergent	15	10	66.7	1	6.7	2	13.3	2	13.3	0	0	13.3

Hypothesis A in this study was that *S. verticillata* plants used as the sources for stem cuttings would produce adventitious roots at different proportions dependent on the source tree height (Table 1). Comparisons among heights were made independently in the cuttings from March and June. No significant differences in rooting proportions of stem cuttings collected from trees of different height categories in either March or in June were detected (Tables 7A, 7B, Figures 15, 16).

Table 7 Comparison among heights. Comparison of root production among stem cuttings collected from different sized trees was analyzed using chi-square (χ^2) analysis of frequencies.

A. Comparison among heights/ages (March). Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Height	No Roots (%)	Roots (%)	Total
Small	60 (60%)	40 (40%)	100
Medium	57 (71%)	23 (29%)	80
Large	146 (66%)	74 (34%)	220
Total	263 (66%)	137 (34%)	400

$\chi^2 = 2.58$ df = 2 P = 0.275

B. Comparison among heights (June). Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Height	No Roots (%)	Roots (%)	Total
Small	79 (79%)	21 (21%)	100
Medium	60 (75%)	20 (25%)	80
Large	173 (79%)	47 (21%)	220
Total	312 (78%)	88 (22%)	400

$\chi^2 = 0.53$ df = 2 P = 0.767

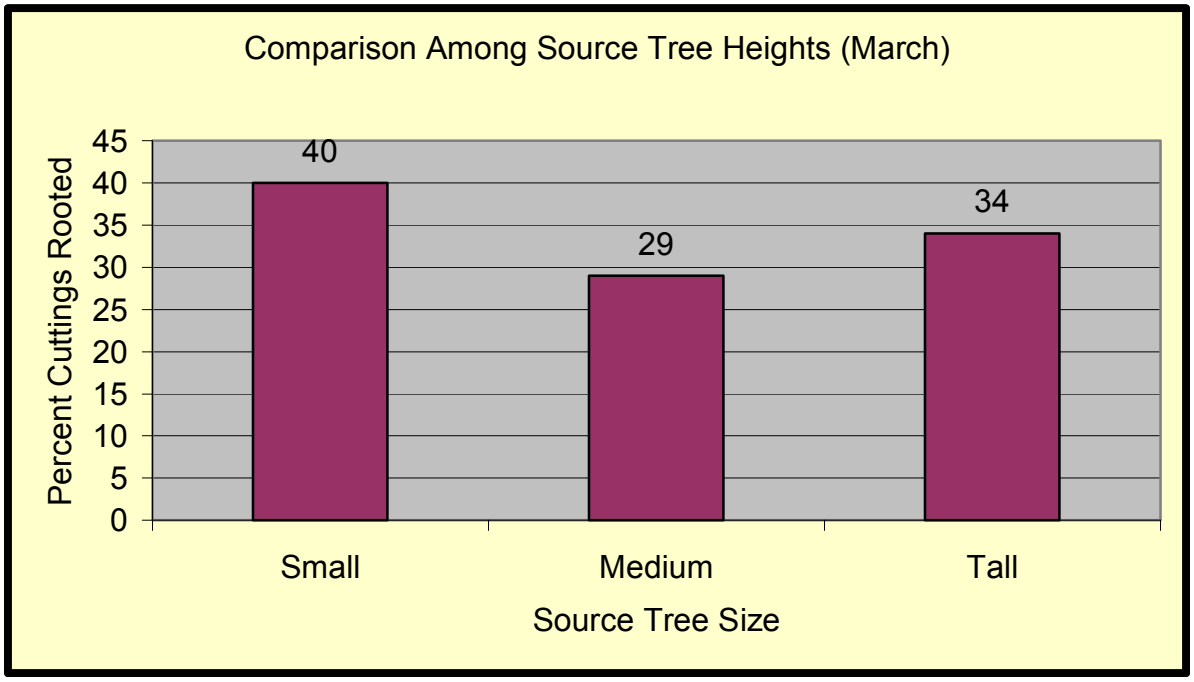


Figure 15 Graphic comparisons among source tree heights (March). Old wood stem cuttings collected in March produced roots at approximately the same proportions. Differences were not significant (data from table 7A).

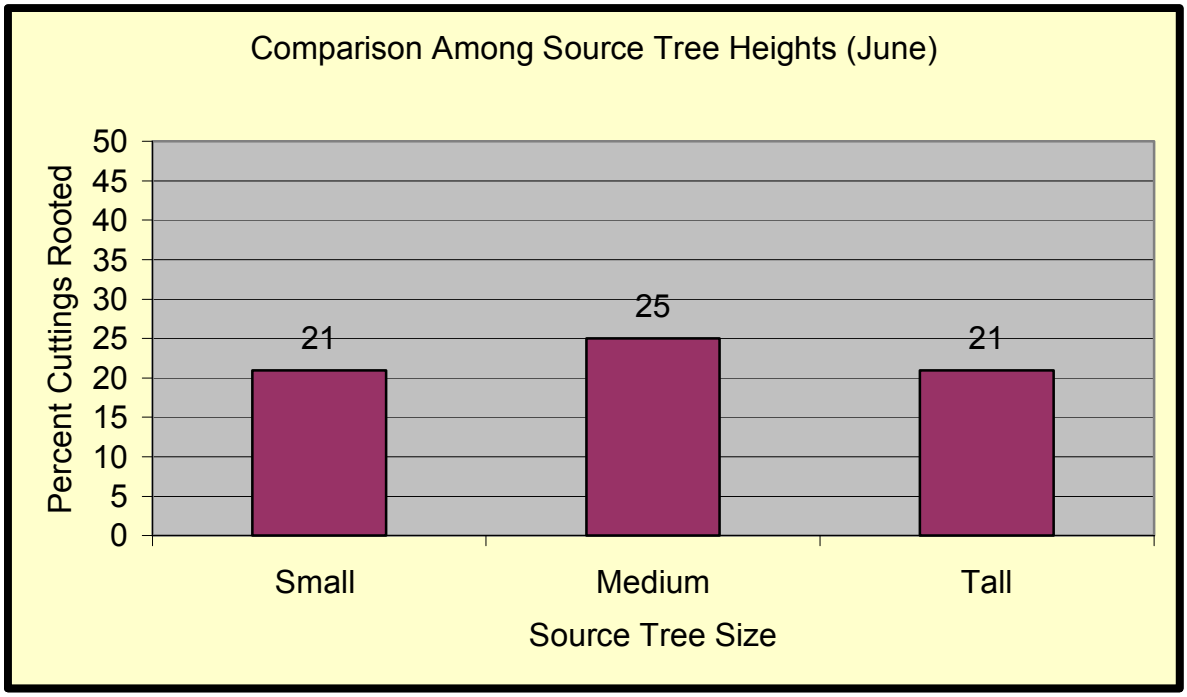


Figure 16 Graphic comparisons among source tree heights (June). New wood stem cuttings collected in June produced roots at approximately the same proportions. Differences were not significant (Data from table 7B).

To test the hypothesis that old wood and new wood cuttings would have different rooting efficiencies, comparison of root production among stem cuttings collected in March and in June were made. There were significant differences in rooting proportions among stem cuttings collected during March (old wood) and June (new wood). Stem cuttings from March rooted at significantly higher proportions than June stem cuttings for small and tall trees; there was no significant difference for medium-sized trees.

Table 8 Test for seasonal effects on rooting of cuttings. Comparisons of root production among stem cuttings collected in March and in June taken from each tree height category.

A. Seasonal effects on rooting for small trees. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	60 (60%)	40 (40%)	100
June	79 (79%)	21 (21%)	100
Total	139 (70%)	61 (30%)	200

$\chi^2 = 8.52$ df = 1 P = 0.004

B. Seasonal effects on rooting for medium trees. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	57 (71%)	23 (29%)	80
June	60 (75%)	20 (25%)	80
Total	117 (73%)	43 (27%)	160

$\chi^2 = 0.29$ df = 1 P = 0.593

C. Seasonal effects on rooting for tall trees. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	146 (26%)	74 (34%)	220
June	173 (79%)	47 (21%)	220
Total	319 (72%)	121 (28%)	440

$\chi^2 = 8.31$ df = 1 P = 0.004

Hypothesis B in this study was that different *S. verticillata* plants used as the source for stem cuttings would produce adventitious roots at different proportions (Table 1). Comparison between trees used as sources of stem cuttings suggested a significant difference in rooting between the individual trees. Further analysis indicated a noteworthy relationship to the sun/shade light environment of the source trees (Tables 9A, 9B). Cuttings collected from trees growing in a shaded site rooted at a greater proportion than the cuttings collected from trees in the sun (Tables 9A, 9B). The differences in rooting proportions were evident in the March cuttings but not in the June cuttings. Cuttings from all trees rooted at a lower proportion in the June cuttings (Tables 9A, 9B, Figures 17, 18). However, inspection of the data showed that within a light environment differences among individual trees were relatively minor. Cuttings collected from sun-grown plants produced roots at approximately the same proportion regardless of the individual or season collected (Tables 9A, 9B). Cuttings collected from shade-grown plants also produced roots at approximately the same proportion regardless of the individual or season collected (Tables 9A, 9B).

Table 9 Comparison between individual trees. Comparison of root production among stem cuttings collected from different individual trees collected in March (old wood) and June (new wood) was analyzed using chi-square (χ^2) analysis of frequencies.

A. Comparison between individual trees (March). Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Tree	No Roots (%)	Roots (%)	Total
VA	97 (81%)	23 (19%)	120
FL	28 (70%)	12 (30%)	40
HC	29 (72%)	11 (28%)	40
LN	29 (72%)	11 (28%)	40
GM (shade)	49 (49%)	51 (51%)	100
MM (shade)	31 (52%)	29 (48%)	60
Total	263 (66%)	137 (34%)	400

$\chi^2 = 31.80$ df = 5 $P < 0.001$

B. Comparison between individual trees (June). Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Tree	No Roots (%)	Roots (%)	Total
VA	103 (86%)	17 (14%)	120
FL	27 (67%)	13 (33%)	40
HC	33 (82%)	7 (18%)	40
LN	31 (77%)	9 (23%)	40
GM (shade)	70 (70%)	30 (30%)	100
MM (shade)	48 (80%)	12 (20%)	60
Total	312 (78%)	88 (22%)	400

$\chi^2 = 11.2$ df = 5 P = 0.047

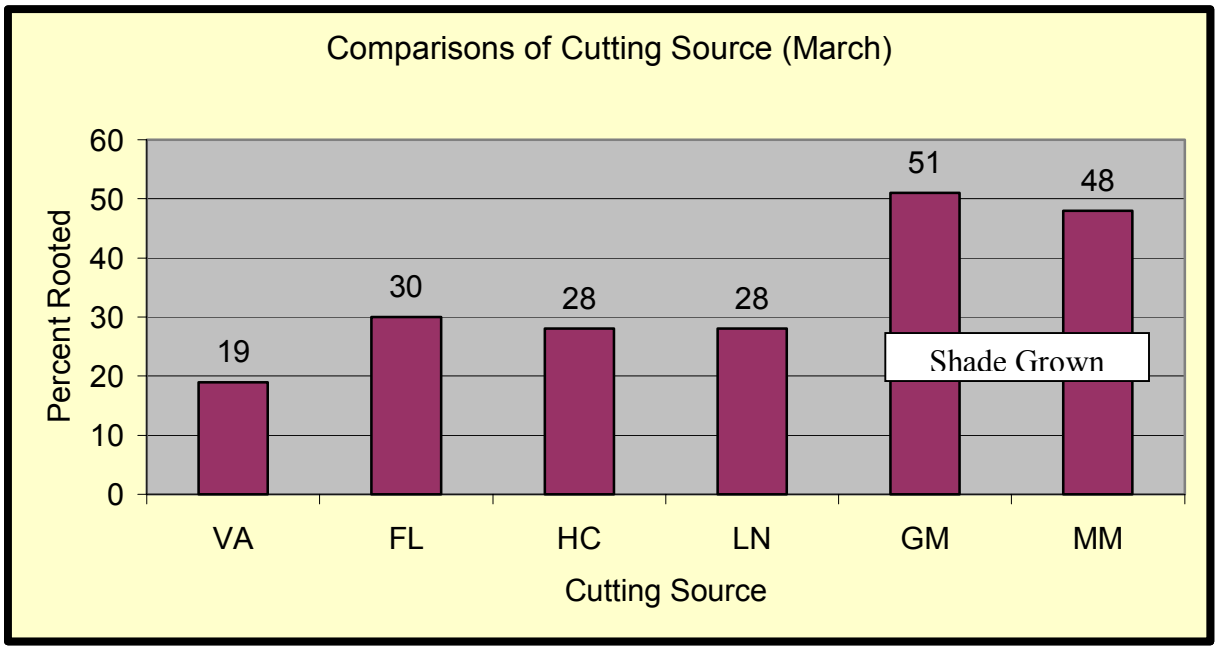


Figure 17 Graphic comparisons between individual trees (March). Graphic representation of comparison of root production between individual trees used as sources of old wood stem cuttings collected in March (data from table 9A).

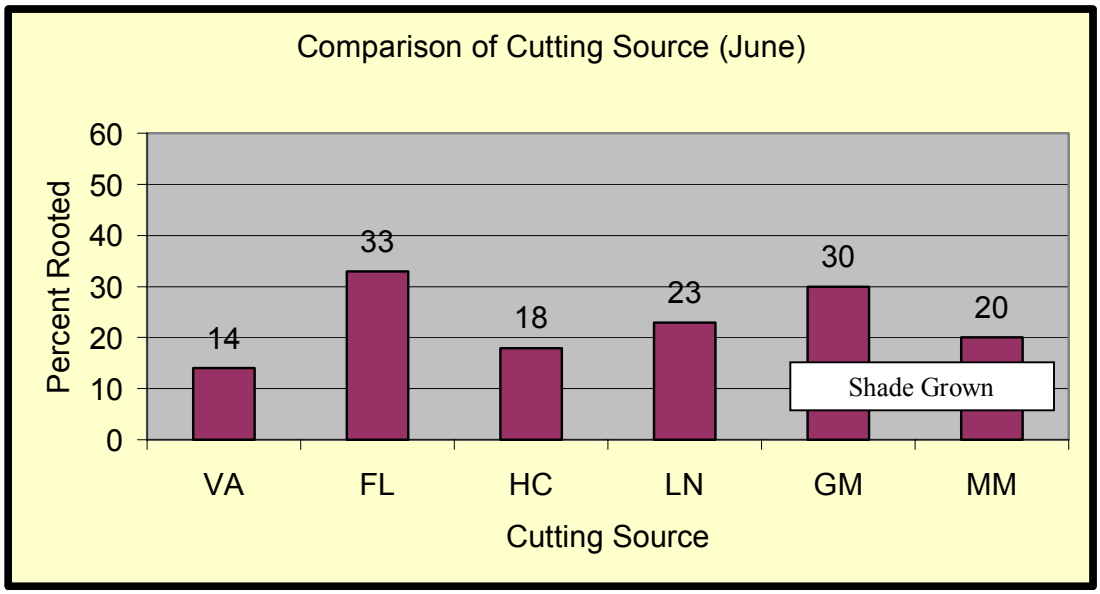


Figure 18 Graphic comparisons between individual trees (June). Graphic representation of comparison of root production between individual trees used as sources of new wood stem cuttings collected in June (data from table 9B).

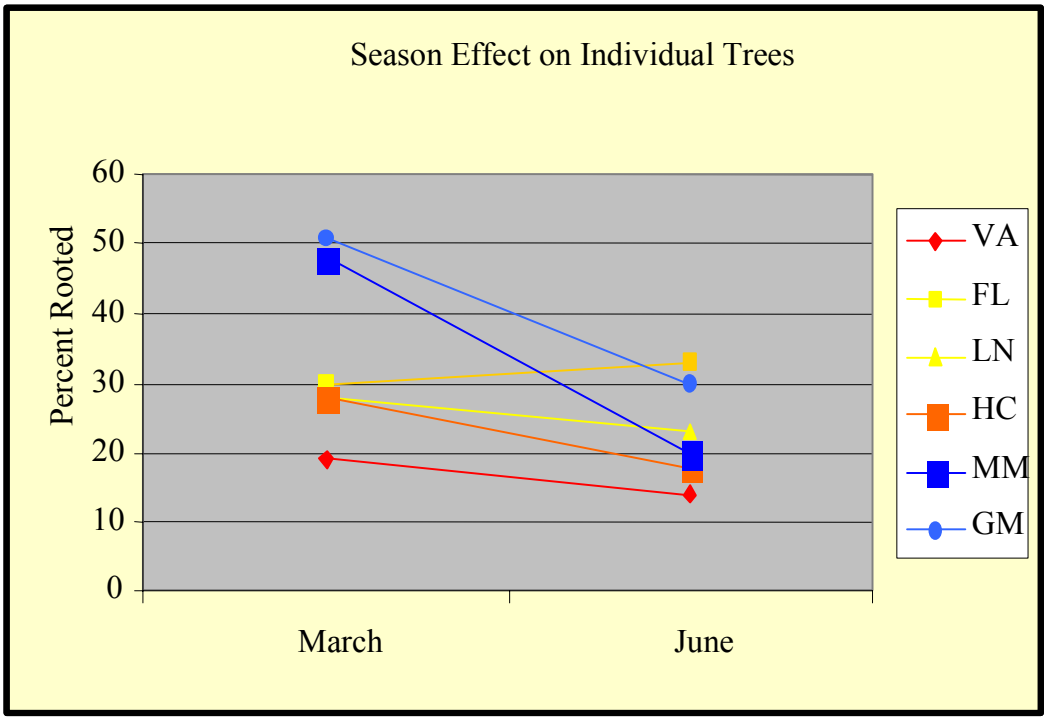


Figure 19 Season effect on individual trees. Graph indicating differences in source tree rooting proportions of stem cuttings between the different seasons. Trees from shade environments are indicated by the blue lines (MM and GM).

Hypothesis C in the current study was that stem cuttings subjected to different treatments would produce adventitious roots at different proportions (Table 1). Comparisons were made independently for March and June trials. Significant differences in rooting between treatments were detected in both the June (new wood) and March (old wood) trials. As previously described, the June cuttings rooted at a lower proportion than the March cuttings and this was consistent within treatments (Table 10A, 10B). Comparisons between treatments indicated an approximate 2-fold increase in rooting of March stem cuttings associated with both water soak treatments as compared to the control and detergent soak treatments (Tables 10A, 10B, Figures 20, 21). Rooting of new wood stem cuttings collected in June was enhanced approximately 2-fold by the 24 hour water soaking pre-treatment and approximately 3-fold by the treatment that included a cut under water when compared to the control and detergent pre-treatments.

Table 10 Comparison between treatments. Comparisons of effectiveness of propagation treatments on production of adventitious roots on stem cuttings of *Sciadopitys verticillata.* Stem cuttings subjected to water soak treatments produced roots at higher proportions than control or detergent water soak treatments.

A. Comparison between treatments (March). Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Treatment	No Roots (%)	Roots (%)	Total
Control	77 (77%)	23 (23%)	100
Water soak	57 (57%)	43 (43%)	100
Cut under water	50 (50%)	50 (50%)	100
Detergent soak	79 (79%)	21 (21%)	100
Total	271 (66%)	139 (34%)	400

$\chi^2 = 27.80$ df = 3 $P < 0.001$

B. Comparison between treatments (June). Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Treatment	No Roots (%)	Roots (%)	Total
Control	87 (87%)	13 (13%)	100
Water soak	74 (74%)	26 (26%)	100
Cut under water	65 (65%)	35 (35%)	100
Detergent soak	86 (86%)	14 (14%)	100
Total	240 (60%)	160 (40%)	400

$\chi^2 = 19.20$ df = 3 $P < 0.001$

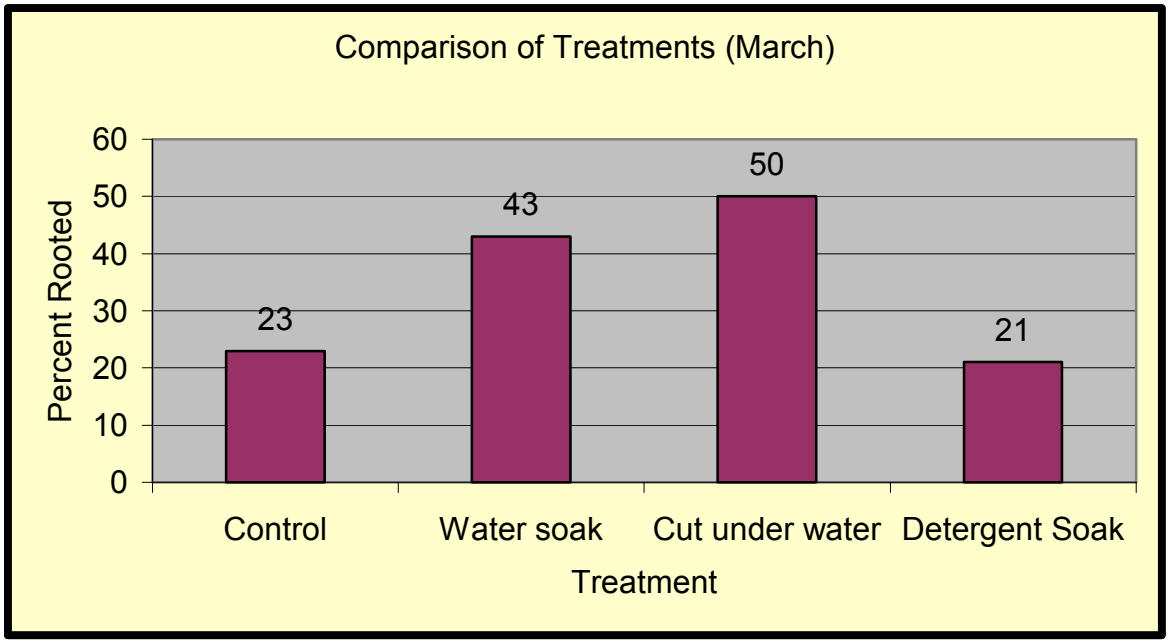


Figure 20 Graphic comparisons between trial pre-treatments (March). Rooting of old wood stem cuttings collected in March was enhanced approximately 2-fold by the fresh water soaking pre-treatments when compared to control and detergent pre-treatments. (data from table 10A).

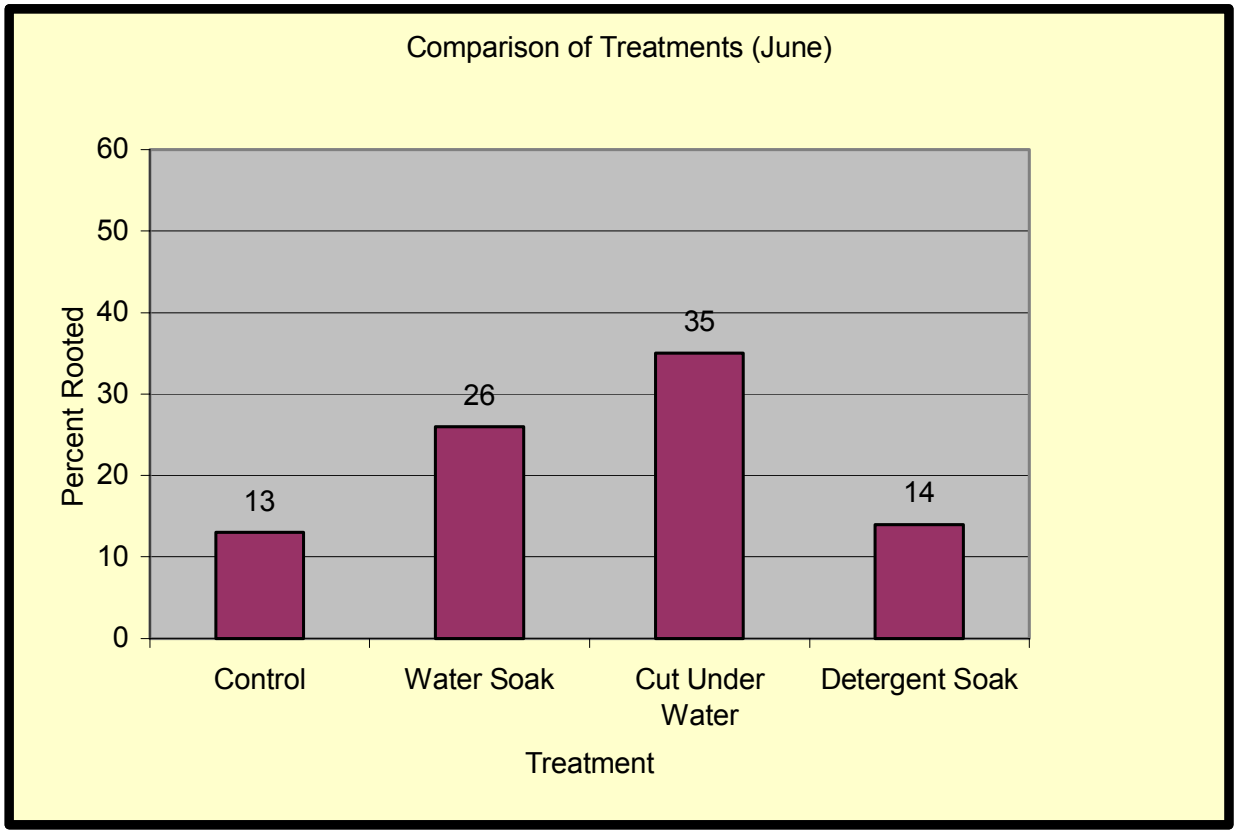


Figure 21 Graphic comparisons between trial pre-treatments (June). Rooting of new wood stem cuttings collected in June was enhanced approximately 2-fold by the 24 hour fresh water soaking pre-treatment and nearly 3-fold by cut under water treatment when compared to control and detergent pre-treatments. (data from table 10B).

Hypothesis D stated that stem cuttings collected during different seasonal stages of growth (summer new wood *vs*. winter old wood) would produce adventitious roots at different proportions (Table 1). There were significant decreases in the proportions of cuttings producing roots in the June trials for 2 of the 3 treatments and the control group had a marginally insignificant difference (Tables 11A, 11B, 11C). The decrease was not statistically significant for the detergent soak treatment (Table 11D).

Table 11 Seasonal effects comparison between treatments

A. Comparison between seasons using data from control treatments. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	77 (77%)	23 (23%)	100
June	87 (87%)	13 (13%)	100
Total	164 (72%)	36 (18%)	200

$\chi^2 = 3.39$ df = 1 P = 0.066

B. Comparison between seasons using data from water soak treatments. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	57 (57%)	43 (43%)	100
June	74 (74%)	26 (26%)	100
Total	131 (65%)	69 (35%)	200

$\chi^2 = 6.39$ df = 1 P = 0.011

C Comparison between seasons using data from cut under water treatments. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	50 (50%)	50 (50%)	100
June	65 (65%)	35 (35%)	100
Total	115 (57%)	85 (43%)	200

$\chi^2 = 4.60$ df = 1 P = 0.032

D. Comparison between seasons using data from detergent soak treatments. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	79 (79%)	21 (21%)	100
June	86 (86%)	14 (14%)	100
Total	93 (46%)	107 (54%)	200

$\chi^2 = 1.70$ df = 1 P = 0.193

There were no a significant difference in the proportion of cuttings that rooted among water soak and cut under water treatments (Tables 12A, 12B).

Table 12 Comparison between soaked in water and cut under water treatments

A. Comparison between soaked in water and cut under water (March). Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Treatment	No Roots (%)	Roots (%)	Total
Soaked in water	57 (57%)	43 (43%)	100
Cut under water	50 (50%)	50 (50%)	100
Total	107 (53%)	93 (47%)	200

$\chi^2 = 0.98$ df = 1 P = 0.321

B. Comparison between soaked in water and cut under water (June). Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Treatment	No Roots (%)	Roots (%)	Total
Soaked in water	74 (74%)	26 (26%)	100
Cut under water	65 (65%)	35 (35%)	100
Total	139 (69%)	61 (31%)	200

$\chi^2 = 1.91$ df = 1 P = 0.167

March (old wood) and June (new wood) cuttings soaked in water treatments were compared. Results showed both water soaking treatments rooted at significantly lower proportions for the June new wood cuttings compared to the March old wood cuttings (Tables 13 and 14).

Table 13 Test for seasonal effects on rooting of cuttings subject to water soak treatments. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	57 (57%)	43 (43%)	100
June	74 (74%)	26 (26%)	100
Total	131 (65%)	69 (35%)	200

$\chi^2 = 6.39$ df = 1 P = 0.011

Table 14 Test for seasonal effects of cuttings subjected to the underwater basal cut treatment. Comparisons were made with dead, callused, and viable categories being classified as 'No Roots'. Both lightly and heavily rooted categories were classified as 'Roots'.

Season	No Roots (%)	Roots (%)	Total
March	50 (50%)	50 (50%)	100
June	65 (65%)	35 (35%)	100
Total	115 (57%)	85 (43%)	200

$\chi^2 = 4.60$ df = 1 P = 0.032

Cuttings from source trees growing in the sun were more likely to form callus than cuttings from shade-grown source trees, but this difference was significant only for old wood cuttings taken in March (Tables 15A, 15B).

Table 15 Test for seasonal differences in frequency of cuttings producing callus and dying. Seasonal comparisons (March *vs.* June) were made independently between the sun and the shade source trees.

A. Seasonal effects comparison of Dead *vs.* Callus of cuttings from trees growing in sun location. Measure: Dead *vs.* Callus

Season	Dead (%)	Callus (%)	Total
March	19 (14%)	116 (86%)	135
June	39 (28%)	99 (72%)	138
Total	58 (21%)	215 (79%)	273

$\chi^2 = 8.21$ df = 1 P = 0.004

B. Seasonal effects comparison of Dead *vs.* Callus of cuttings from trees growing in shade location. Measure: Dead *vs.* Callus

Season	Dead (%)	Callus (%)	Total
March	54 (86%)	9 (14%)	63
June	70 (90%)	8 (10%)	78
Total	124 (88%)	17 (12%)	141

$\chi^2 = 0.53$ df = 1 P = 0.465

Cuttings collected from all trees, except the "FL" tree (ID #2), rooted at a higher proportion in the March old wood trial compared to the June new wood trial (Tables 16A, 16B, 16C, 16D, 16E, 16F). The differences were not significant among the sun-grown trees (VA, FL, LN, and HC), but there was a significant decrease observed in the rooting proportions of cuttings from shade grown trees (MM and GM) (Tables 16A, 16B, 16C, 16D, 16E, 16F).

Table 16 Test for source tree-specific seasonal effects on rooting of cuttings

A. Test for seasonal effects of stem cuttings producing roots (VA tree)

Season	No Roots (%)	Roots (%)	Total
March	97 (81%)	23 (19%)	120
June	103 (86%)	17 (14%)	120
Total	200 (83%)	40 (17%)	240

$\chi^2 = 1.08$ df = 1 P = 0.299

B. Test for seasonal effects of stem cuttings producing roots (FL tree)

Season	No Roots (%)	Roots (%)	Total
March	28 (70%)	12 (30%)	40
June	27 (67%)	13 (33%)	40
Total	55 (69%)	25 (31%)	80

$\chi^2 = 0.58$ df = 1 P = 0.809

C. Test for seasonal effects of stem cuttings producing roots s (LN tree)

Season	No Roots (%)	Roots (%)	Total
March	29 (72%)	11 (28%)	40
June	31 (77%)	9 (23%)	40
Total	60 (75%)	20 (25%)	80

$\chi^2 = 0.27$ df = 1 P = 0.606

D. Test for seasonal effects of stem cuttings producing roots (HC tree)

Season	No Roots (%)	Roots (%)	Total
March	29 (72%)	11 (28%)	40
June	33 (82%)	7 (18%)	40
Total	62 (77%)	18 (23%)	80

$\chi^2 = 1.15$ df = 1 P = 0.284

E. Test for seasonal effects of stem cuttings producing roots (MM tree)

Season	No Roots (%)	Roots (%)	Total
March	31 (52%)	29 (48%)	60
June	48 (80%)	12 (20%)	60
Total	79 (66%)	41 (34%)	120

$\chi^2 = 10.70$ df = 1 $P < 0.001$

F. Test for seasonal effects of stem cuttings producing roots (GM tree)

Season	No Roots (%)	Roots (%)	Total
March	49 (49%)	51 (51%)	100
June	70 (70%)	30 (30%)	100
Total	119 (59%)	81 (41%)	200

$\chi^2 = 9.15$ df = 1 $P = 0.002$

Hypothesis E was that stem cuttings collected from trees growing in different light (sun *vs*. shade) environments would produce adventitious roots at different proportions. Stem cuttings collected from trees growing in shade rooted more successfully than stem cuttings collected from trees growing in sun (Tables 17A, 17B). March old wood cuttings taken from shaded trees rooted at significant higher proportions than cuttings taken from sun located trees, producing roots at twice the proportion of the cuttings collected from source trees in the sun (Table 17A, Figure 22). The difference in rooting of new wood (June) cuttings from shade and sun-grown source trees was not significant (Table 17B, Figure 23).

Table 17 Test for effects of source tree light environment on rooting of cuttings

A. Effect of light environment of source tree (March)

Source Tree Environment	No Roots (%)	Roots (%)	Total
Sun	181 (75%)	59 (25%)	240
Shade	80 (50%)	80 (50%)	160
Total	261 (65%)	139 (35%)	400

$\chi^2 = 27.35$ df = 1 $P < 0.001$

B. Effect of light environment of source tree (June)

Source Tree Environment	No Roots (%)	Roots (%)	Total
Sun	194 (81%)	46 (19%)	240
Shade	118 (74%)	42 (26%)	160
Total	312 (78%)	88 (22%)	400

$\chi^2 = 2.81$ df = 1 P = 0.094

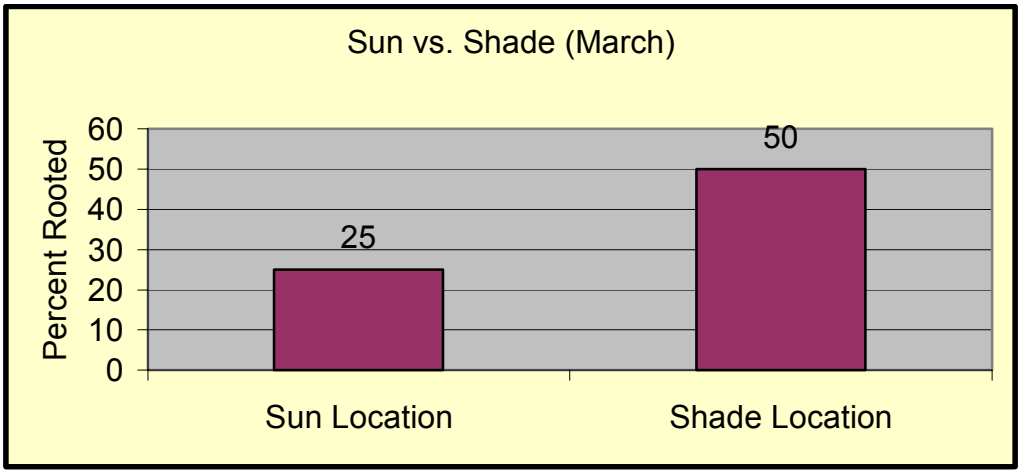


Figure 22 Graphical representation of test for effects of source tree light environment on rooting of cuttings (March). Old wood stem cuttings collected from shade-grown trees in March produced roots at twice the proportion of the sun-grown trees (data from table 17A).

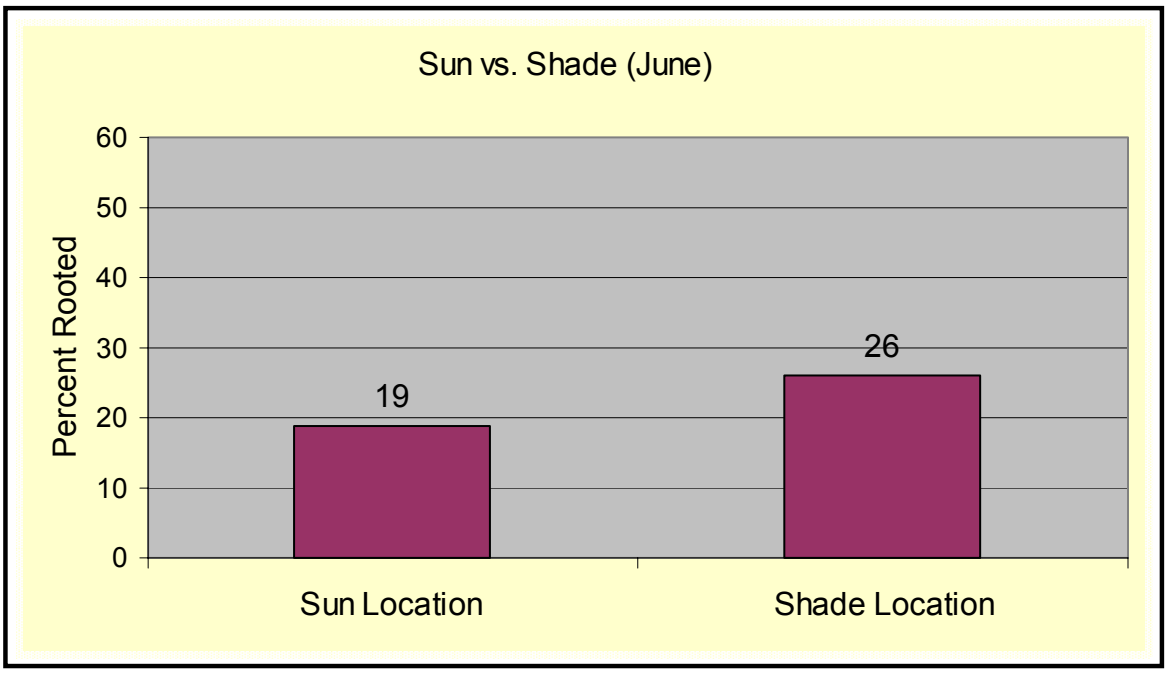


Figure 23 Graphical representation of test for effects of source tree light environment on rooting of cuttings (June). The difference was not significant (data from table 17B).

Old wood cuttings had a significantly higher proportion of cuttings producing roots from trees growing in a shade environment than from trees growing in a sun environment regardless of source tree age/size or cutting treatment (Tables 17A, 18A, 18B). There were no significant seasonal differences in rooting of stem cuttings collected from sun-grown trees (Table 17B, Figure 24). Old wood cuttings collected in March rooted twice as effectively as did the new wood stem cuttings collected in June (Figure 25). Cuttings collected from sun-grown trees rooted at lower proportions regardless of season. The differences in rooting were less evident in the June trials but the cuttings from shade-grown trees rooted at a greater proportion than did the cuttings from sun-grown trees (Tables 17B, 18A, 18B, 24). The difference in rooting of new wood (June) and old wood (March) cuttings from sun-grown source trees was not significant (Tables 18A, 18B) (Figures 24, 25, 26).

Table 18 Test for seasonal effects on rooting of cuttings. Comparisons were made independently between sun-grown and shade-grown source trees. Old wood stem cuttings collected in March rooted at higher proportions than new wood stems collected in June regardless of sun/shade location.

A. Seasonal effects on rooting of stem cuttings from source trees in the sun

Season	No Roots (%)	Roots (%)	Total
March cuttings	181 (75%)	59 (25%)	240
June cuttings	194 (81%)	46 (19%)	240
Total	375 (78%)	105 (22%)	480

$\chi^2 = 2.06$ df = 1 P = 0.151

B. Seasonal effects on rooting of stem cuttings from source trees in the shade

Season	No Roots (%)	Roots (%)	Total
March cuttings	80 (50%)	80 (50%)	160
June cuttings	118 (74%)	42 (26%)	160
Total	198 (62%)	122 (38%)	320

$\chi^2 = 19.10$ df = 1 P < 0.001

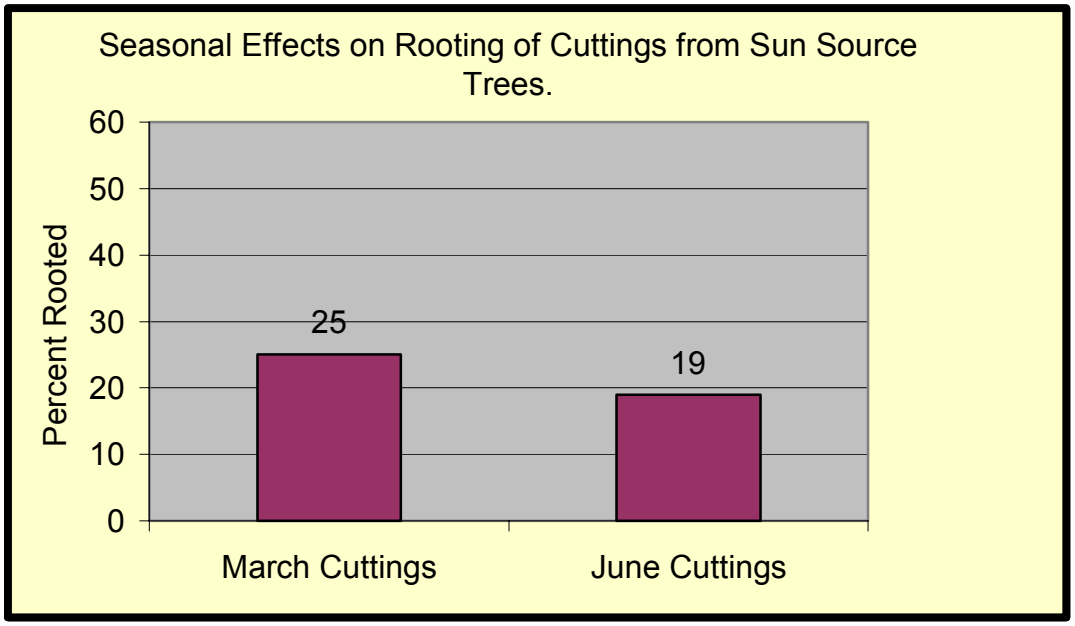


Figure 24 Graphical representation of test for seasonal effects on rooting of cuttings from sun source trees. There were no significant seasonal differences in stem cuttings collected from sun-grown trees. Sun location stem cuttings rooted at low proportions regardless of season, although this difference was not statistically significant (data from table 18A).

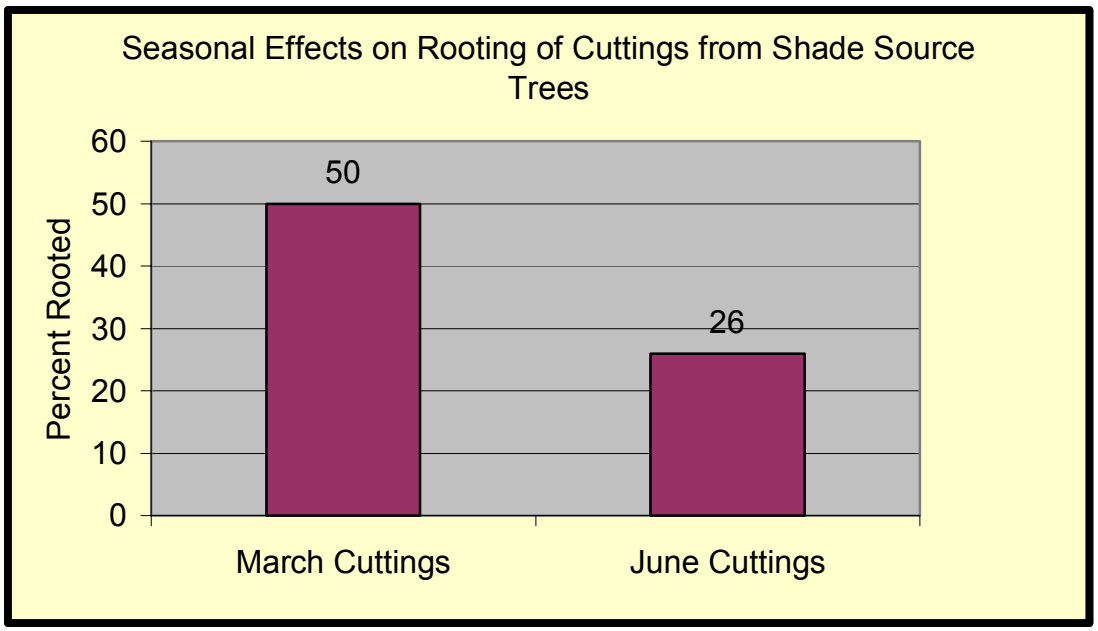


Figure 25 Graphical representation of test for seasonal effects on rooting of cuttings collected from shade source trees. Old wood cuttings collected in March rooted twice as effectively as did the new wood stem cuttings collected in June (data from table 18B).

The light environment of the source trees had a greater effect on the rooting proportions of old wood stem cuttings collected in March than on new wood stem cuttings collected in June. Old wood stem cuttings collected in March rooted at higher proportions than new wood stems collected in June regardless of sun/shade location, although a significant difference was only seen in cuttings from shade source trees (Figure 26).

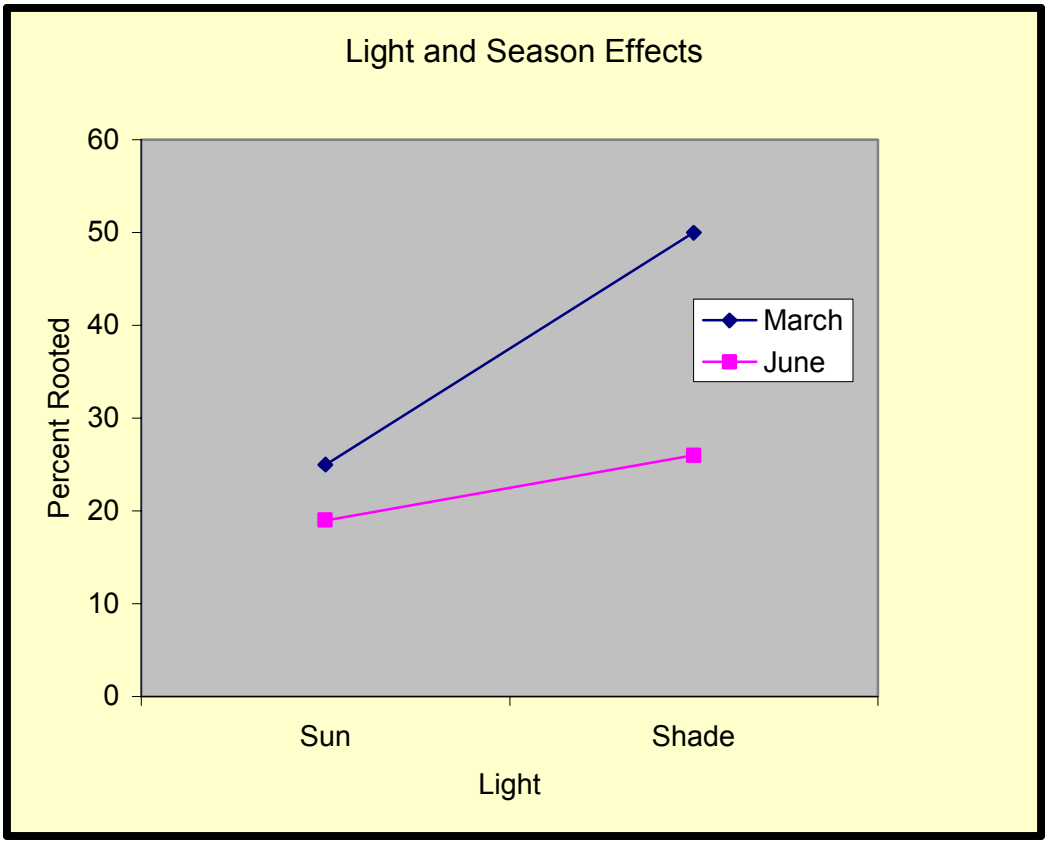


Figure 26 Graph of light effect of source tree on stem cuttings collected in March and June. Sun/shade environment had a greater effect on the rooting proportions of old wood stem cuttings collected in March than on new wood stem cuttings collected in June.

Stem cuttings from source trees growing in a sunny location produced callus at significantly higher proportions than stem cuttings from source trees growing in a shady location in both the March and June trials. Stem cuttings from source trees growing in a shaded location died at significant higher proportions than stem cuttings from source trees growing in a sunny

location in both the March and June trials. Regardless of the season, a significantly higher proportion of cuttings produced callus from trees growing in the sun compared to trees growing in the shade (Tables 19A, 19B). If a stem cutting did not produce roots, it either died or produced callus. Callus was produced on stem cuttings at a rate 6 times higher for source trees growing in sun locations than source trees growing in shade locations (Figure 27).

Table 19 Comparison of number of dead *vs.* callus-producing cuttings taken from source trees growing in sun and shade locations. Comparisons were made independently for cuttings taken in March and June.

A. Comparison of number of dead *vs.* callus-producing cuttings (March)

Source Tree Environment	Dead (%)	Callus (%)	Total
Sun	19 (14%)	116 (86%)	135
Shade	54 (86%)	9 (14%)	63
Total	73 (37%)	125 (63%)	198

$\chi^2 = 94.70$ df = 1 $P < 0.001$

B. Comparison of number of dead *vs.* callus-producing cuttings (June)

Source Tree Environment	Dead (%)	Callus (%)	Total
Sun	39 (28%)	99 (72%)	138
Shade	70 (90%)	8 (10%)	78
Total	109 (51%)	107 (49%)	216

$\chi^2 = 75.4$ df = 1 $P < 0.001$

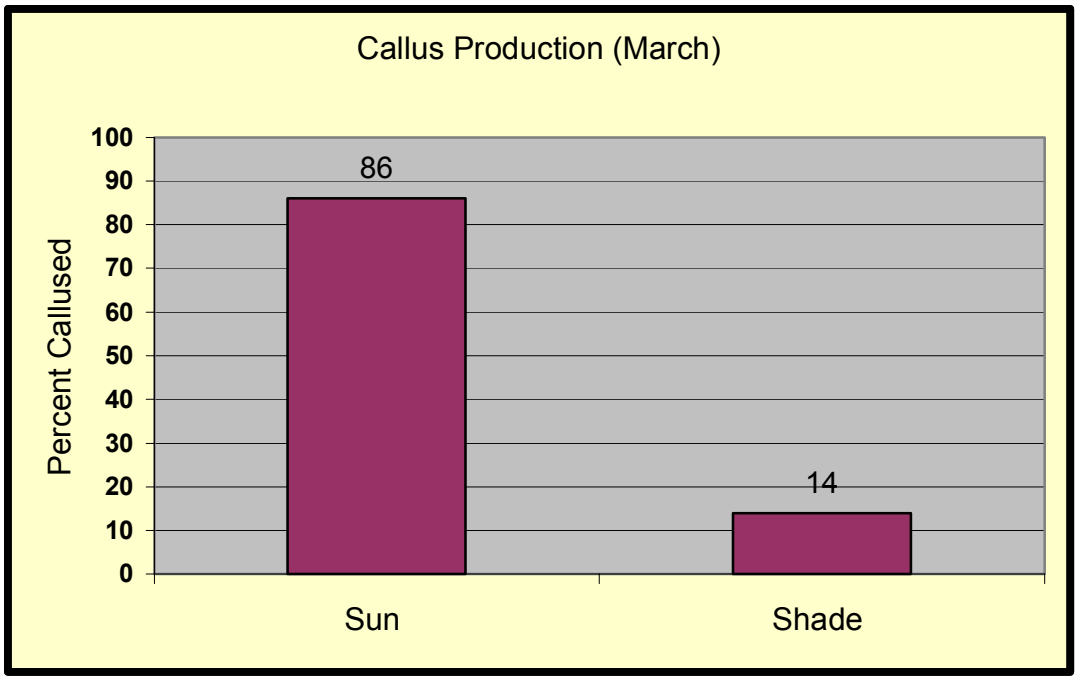


Figure 27 Graphic comparison of callus production between sun and shade source trees (March). If the stem cutting did not produce roots they either died or produced callus. Callus was produced on stem cuttings at proportions 6 times higher among source trees growing in sun than source trees growing in shade (data from table 11A).

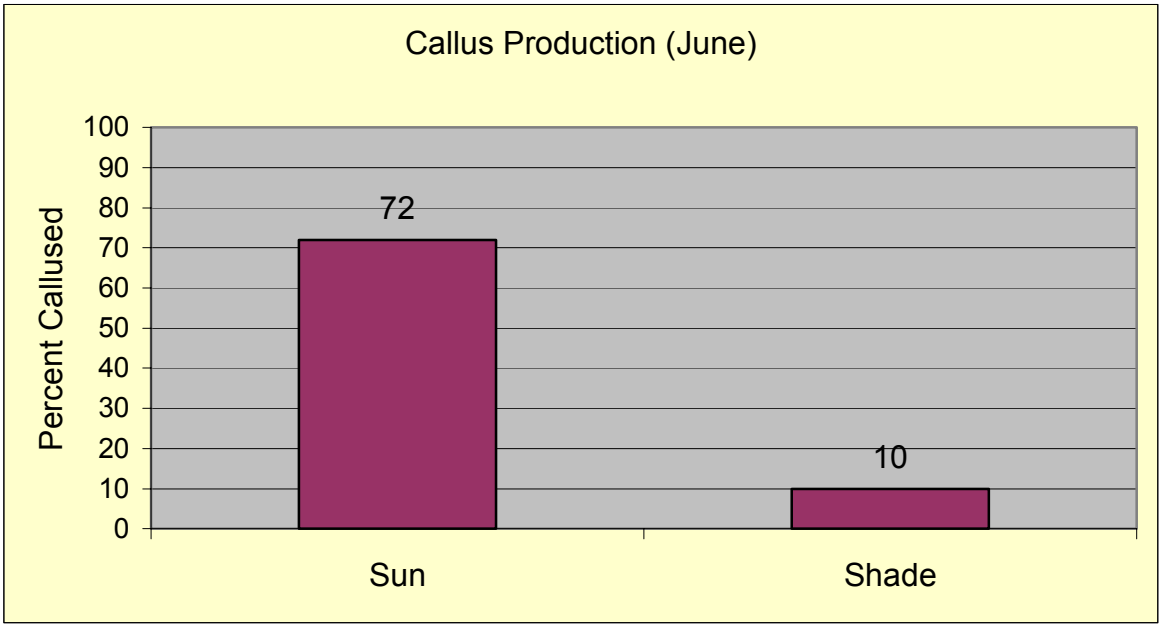


Figure 28 Graphic comparison of callus production between sun and shade source trees (June). If the stem cutting did not produce roots they either died or produced callus. Callus was produced on stem cuttings at proportions 7 times higher among source trees growing in sun than source trees growing in shade (data from table 11B).

Antibiotic Trials

The presence or absence of bacterial inhibition zones induced by *S. verticillata* sap extracts is shown in Table 5. An "X" indicates that there was no inhibition of bacteria growth. A "√" indicates inhibition of bacteria growth, visualized as an inhibition area as large or larger than the sap treatment droplet. A "√-" indicates some inhibition of bacteria growth that was visualized as an inhibition area smaller than the applied sap treatment droplet or incomplete inhibition where some bacterial colonies were growing within the treated area. "NC" indicates that the treatment was not conducted.

Table 20 Effect of *S. verticillata* sap treatments on growth of bacterial species. "X" indicates no inhibition of bacteria growth, "√" indicates total inhibition of bacteria growth, and"√-" indicates some inhibition of bacteria growth. R.T. = room temperature sap/aqueous NaCl mixture; 65 C – 80 C = room temperature sap mixture heated to 65 - 80 C.; 100 C = room temperature sap mixture heated to 100 C.; Super = supernatant from centrifuging of sap mixture; Latex = latex pellet from centrifuging of sap mixture; 100:1 = 1 part latex diluted with 100 parts NaCl.

Bacterial Species	Gram (+ or -)	Control NaCl	R.T. Sap	65 C – 80 C	100 C	Super	Latex	100:1
Staphylococcus aureus	+	X	√	√	NC	X	√	√
Staphylococcus epidermidis	+	X	√	√	NC	X	√	NC
Moraxella catarrhalis	-	X	√	√	√	X	√	√-
Neisseria cinerea	-	X	√	√	√	X	√	√-
Bacillus cereus	+	X	√	√	NC	X	√	NC
Bacillus subtilis	+	X	√	√	NC	X	√	NC
Escherichia coli	-	X	X	X	NC	X	X	NC
Agrobacterium tumefaciens	-	X	X	X	X	X	X	X
Pseudomonas syringae	-	X	X	X	X	X	X	X
Xanthomonas species	-	X	X	X	X	X	X	X
Erwinia amylovora	-	X	X	X	X	X	X	X

There were no inhibition zones visible in any control sectors or supernatant sectors. The latex-like sap inhibited some bacterial species but not others, with no evident pattern of inhibition among Gram-positive or Gram-negative bacterial species. None of the 4 plant pathogens were inhibited, but inhibition of growth of the soil bacilli, staphylococci, *Moraxella catarrhalis* and *Neisseria cinerea* was observed (Table 20).

There were clear inhibition zones visible on plates growing *Staphylococcus aureus* (Figures 29, 31, 32), *Staphylococcus epidermidis* (Figure 30, 31), *Moraxella catarrhalis* (Figure 33), *Neisseria cinerea* (Figure 34), *Bacillus cereus* (Figure 35), and *Bacillus subtilis* (Figure 36) treated with the room temperature mixture, the concentrated latex, and the heat-treated extracts. *Moraxella catarrhalis* and *Neisseria cinerea* were also inhibited by sap extracts heated to 100 C.

Clear inhibition zones were visible on plates growing *Staphylococcus aureus* at the 100:1 dilution of the sap mixture. *Staphylococcus epidermidis* and *Neisseria cinerea* were partially inhibited at the 100:1 dilution (Table 20).

There were no inhibition zones visible on any of the plates containing *Escherichia coli* (Figure 37), *Pseudomonas syringae* (Figures 41 - 43), *Agrobacterium tumefaciens* (Figures 38 - 40), *Xanthomonas sp.* (Figures 43 - 45), or *Erwinia amylovora* (Figures 46, 47) using any of the treatments.

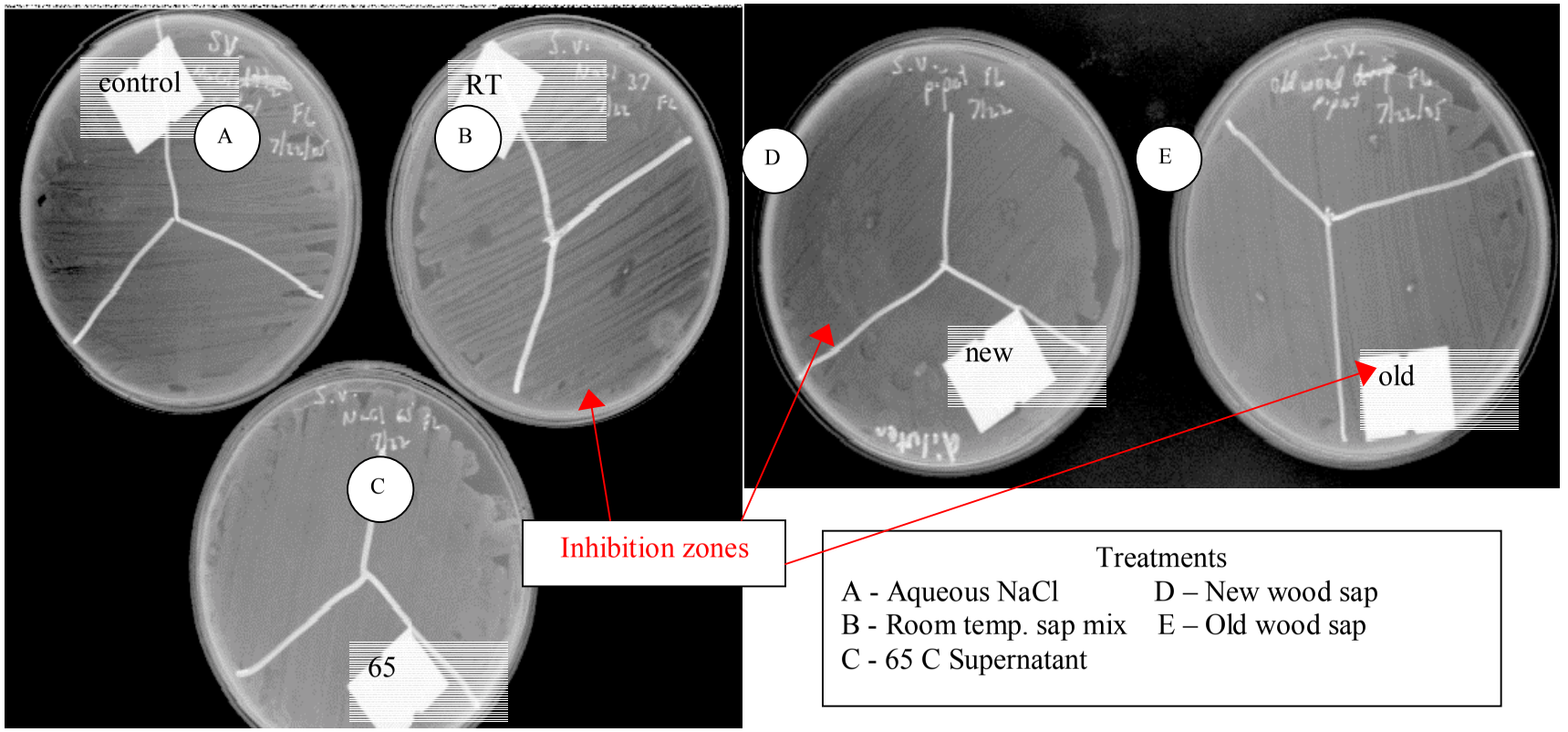


Figure 29 Preliminary *Staphylococcus aureus* antibiotic trial. Petri dishes containing *S. aureus* subjected to 5 different treatments. Each dish had 3 treatment drops applied, 1 per sector. Small inhibition zones were observed in the room temperature (RT) sap mixture, new wood sap and old wood sap trials. No inhibition zones were detected in control or 65 C trial. This trial was the first indication that *S. verticillata* sap inhibited bacterial growth.

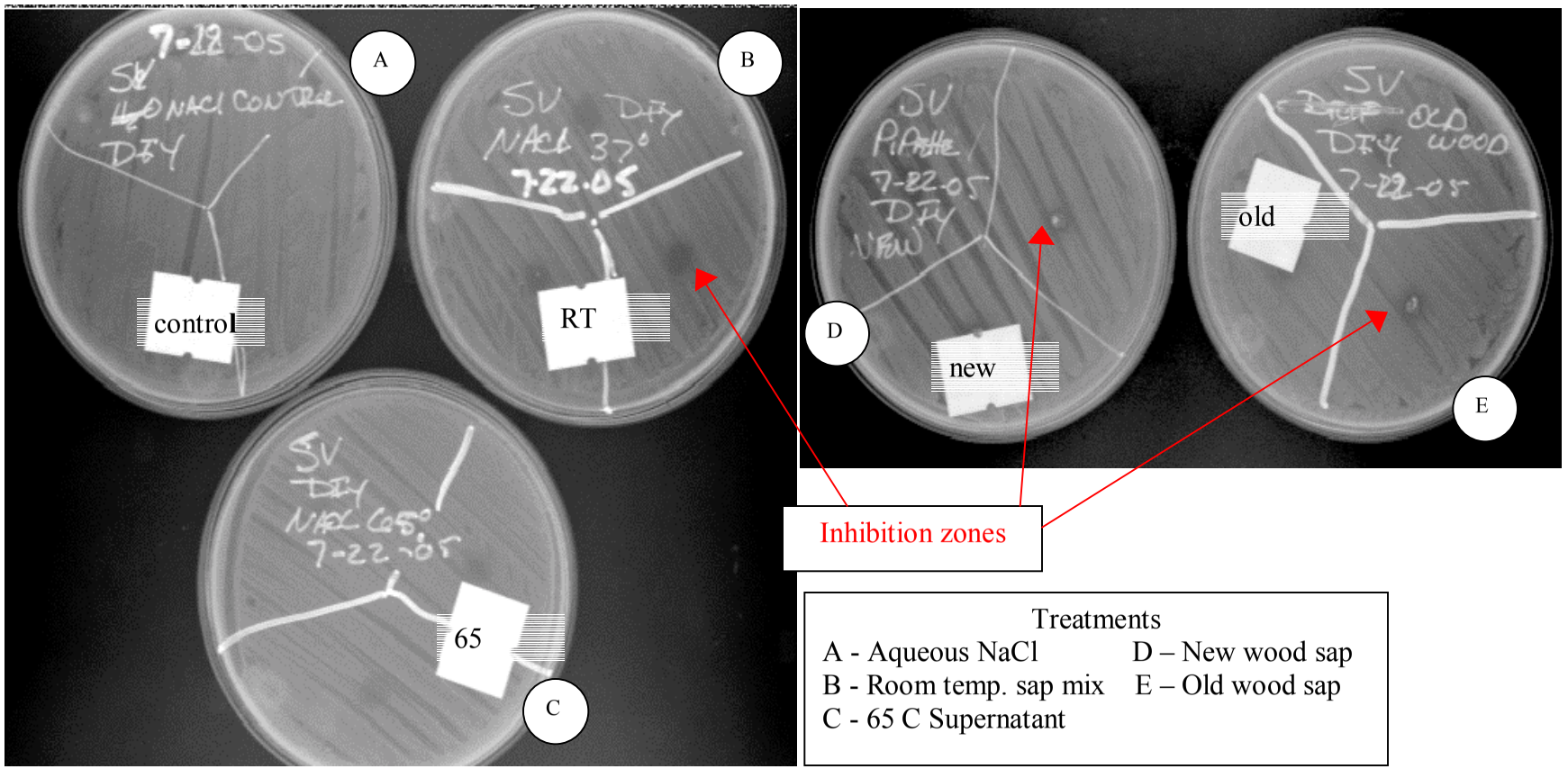


Figure 30 Preliminary *Staphylococcus epidermidis* antibiotic trial. Petri dishes containing *S. epidermidis* subjected to 5 different treatments. Each dish had 3 treatment drops applied, 1 per sector. Small inhibition zones were observed in the room temperature (RT) sap mixture, new wood sap, and old wood sap trials. No inhibition zones were detected in control (NaCl) or 65 C trial.

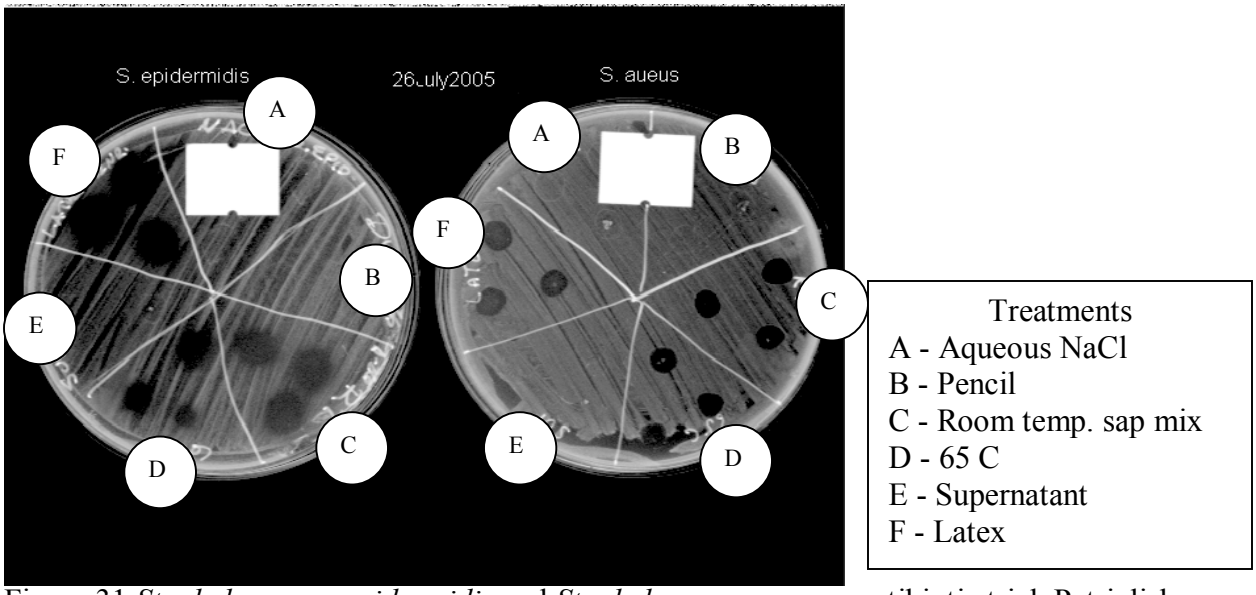


Figure 31 *Staphylococcus epidermidis* and *Staphylococcus aureus* antibiotic trial. Petri dishes containing *S. epidermidis* and *S. aureus* subjected to 6 different treatments. Each dish had 3 treatment drops per sector. Clear inhibition zones were observed in room temperature (RT) sap mixture, 65 C, and latex sectors. No inhibition zones were detected in control (NaCl), supernatant, or pencil sectors. Sap extraction and application procedures improved and clearer inhibition zones were more visible in this trial than in preliminary trials (Figures 29, 30).

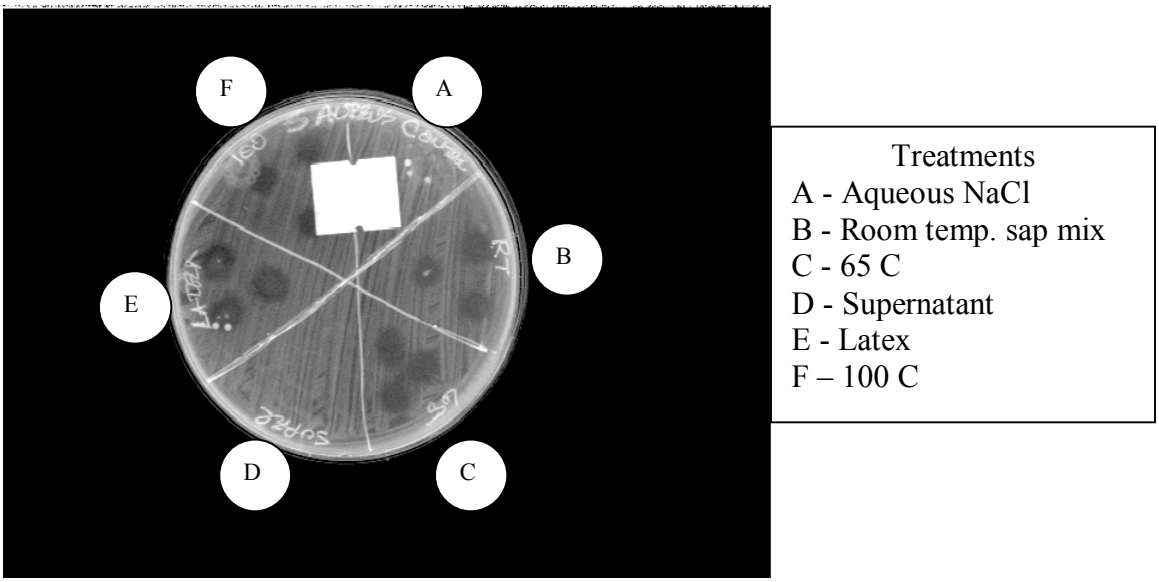
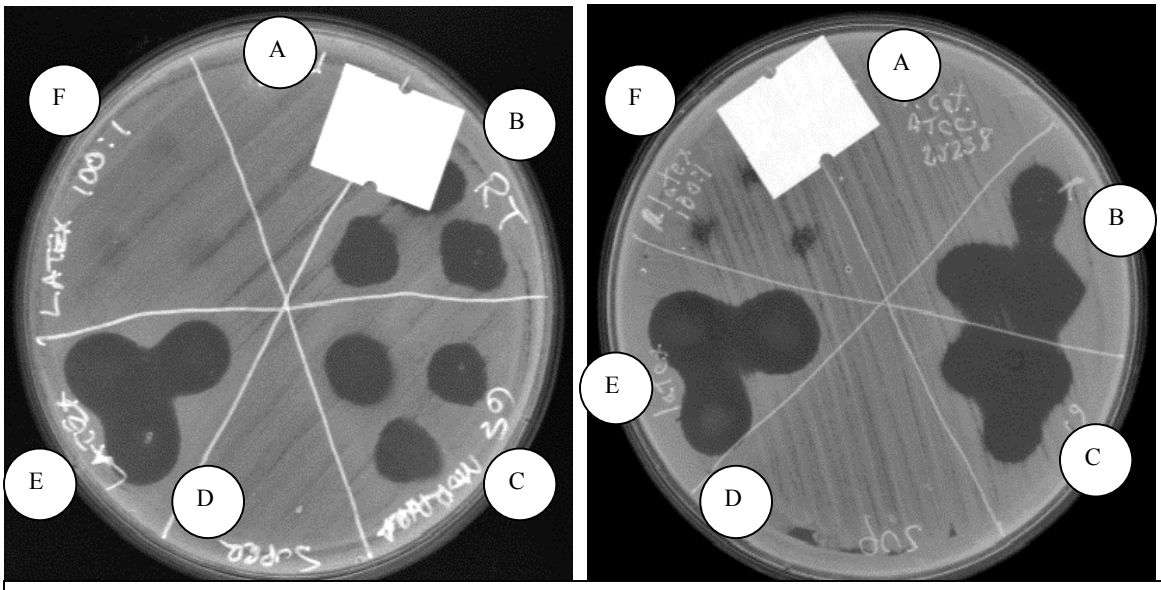
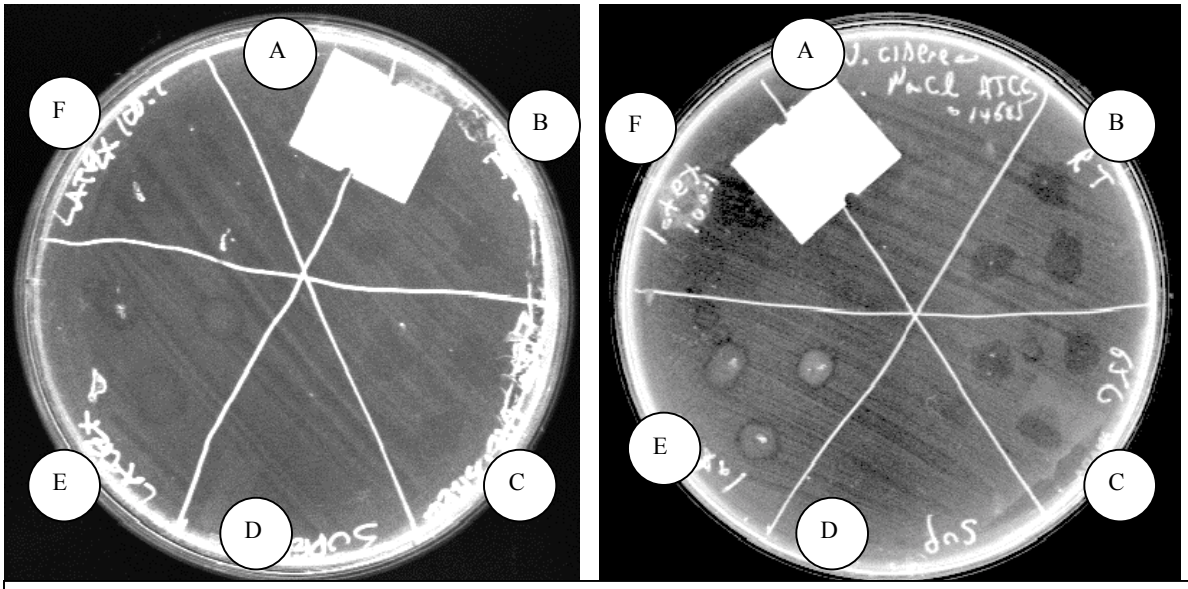


Figure 32 *Staphylococcus aureus* antibiotic trials. Petri dishes containing *Staphylococcus aureus* subjected to 5 different treatments. Three treatment drops were applied per sector. Clear inhibition zones were observed in room temperature (RT) sap mixture, 65 C, 100 C, and latex sectors. No inhibition zones were detected in control (NaCl) or supernatant sectors.



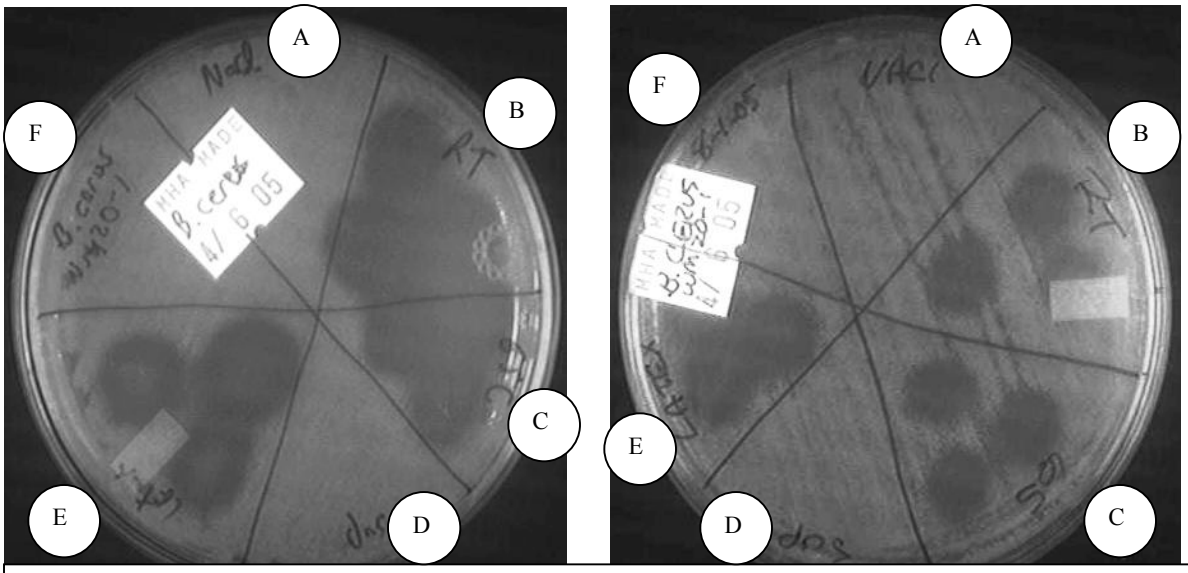
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D – Supernatant, E – Latex, F – Latex 100:1

Figure 33 *Moraxella catarrhalis* antibiotic trials. Petri dishes containing *M. catarrhalis* subjected to 6 different treatments. Three treatment drops were applied per sector. Clear inhibition zones were observed in room temperature (RT) sap mixture, 65 C, and latex sectors. Some bacterial inhibition was observed in latex the 100:1 sector. No inhibition zones were detected in control (NaCl) or supernatant sectors.



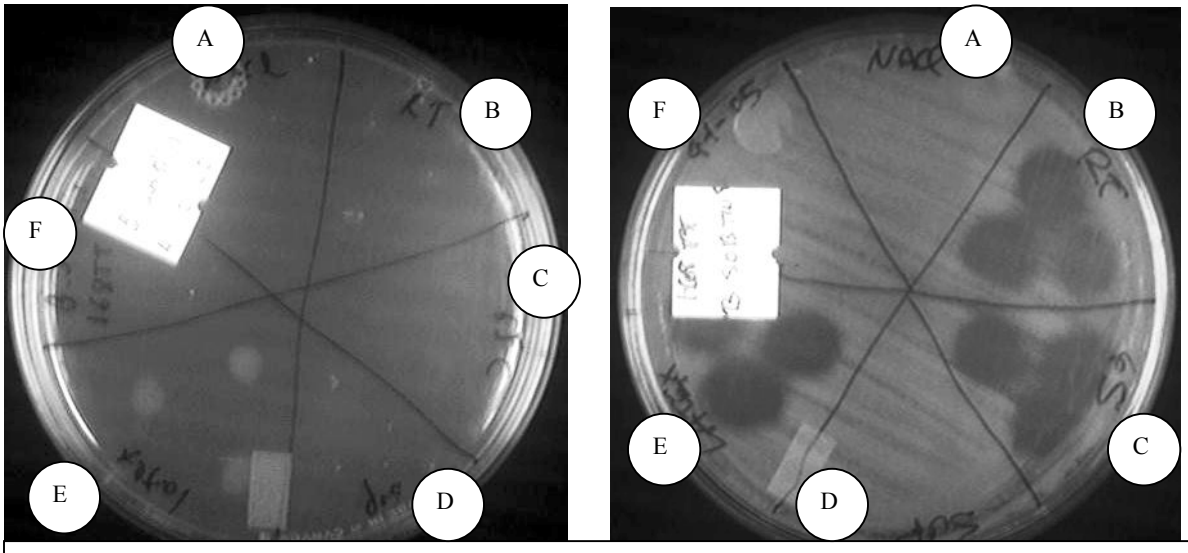
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D – Supernatant, E – Latex, F – Latex 100:1

Figure 34 *Neisseria cinerea* antibiotic trials. Petri dishes containing *Neisseria cinerea* subjected to 6 different treatments. Three treatment drops were applied per sector. Inhibition zones were observed in room temperature (RT) sap mixture, 65 C, and latex sectors. Some bacterial inhibition was observed in latex the100:1 sector. No inhibition zones were detected in control (NaCl) or supernatant sectors.



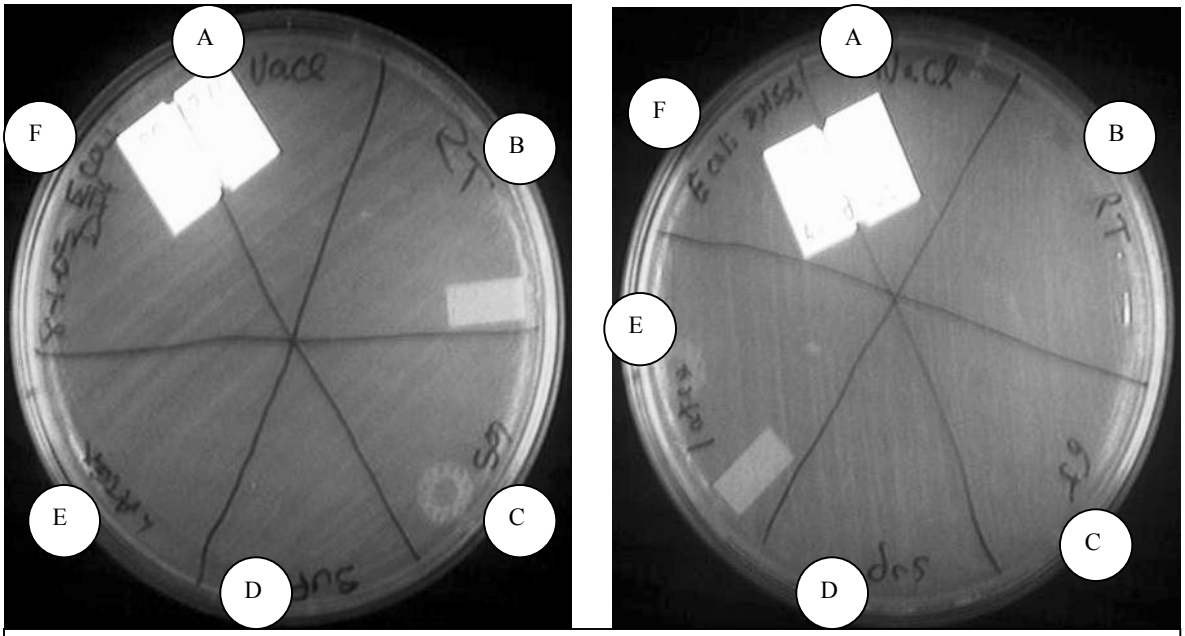
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D – Supernatant, E – Latex, F – Empty

Figure 35 *Bacillus cereus* antibiotic trials. Petri dishes containing *B. cereus* subjected to 5 different treatments. Three treatment drops were applied per sector. Clear inhibition zones were observed in room temperature (RT) sap mixture, 65 C, and latex sectors. No inhibition zones were detected in control (NaCl) or supernatant sectors. No treatment was applied to sector F.



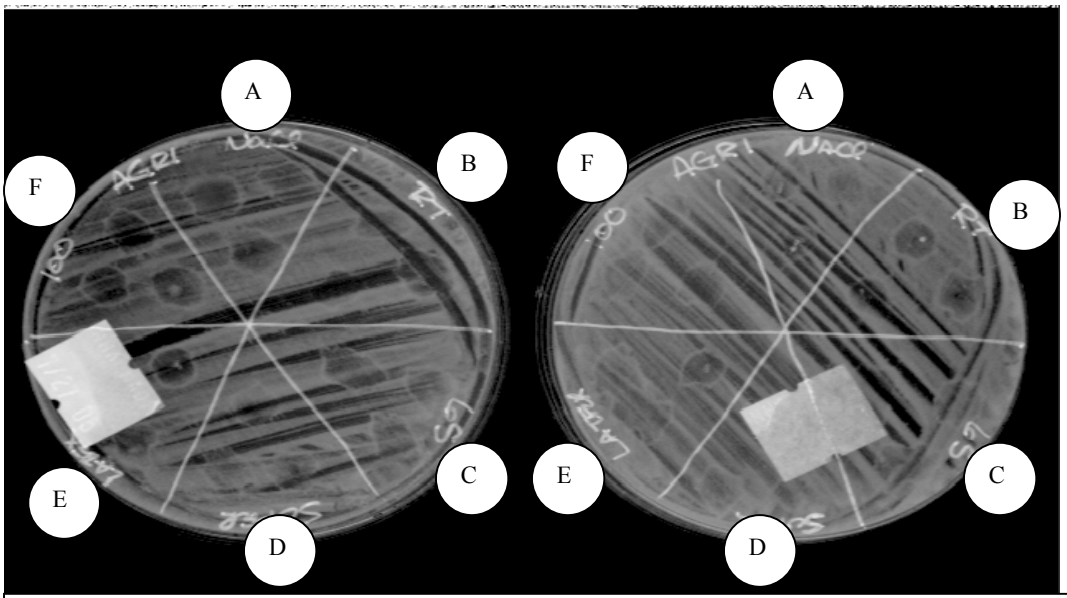
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D – Supernatant, E – Latex, F – Empty

Figure 36 *Bacillus subtilis* antibiotic trials. Petri dishes containing *B. subtilis* subjected to 5 different treatments. Three treatment drops were applied per sector. Bacterial growth was absent from the left dish, likely due to improper laboratory procedure. In the right dish, clear inhibition zones were observed in applied room temperature (RT) sap mixture, 65 C, and latex sectors. No inhibition zones were detected in control (NaCl) or supernatant sectors. No treatment was applied to sectors labeled F.



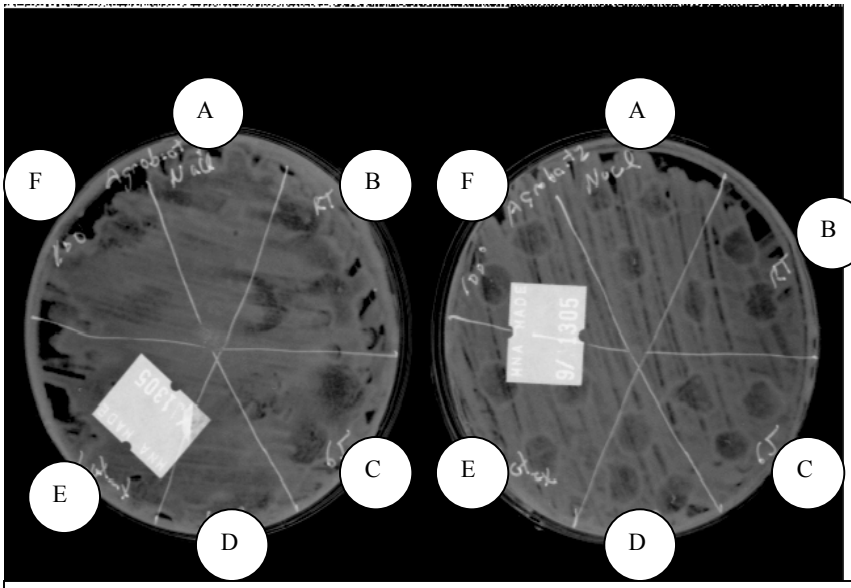
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D - Supernatant, E - Latex,

Figure 37 *Escherichia coli* antibiotic trials. Petri dishes containing *E. coli* subjected to 5 different treatments. No inhibition zones were detected in any sector. No treatment was applied to sector F.



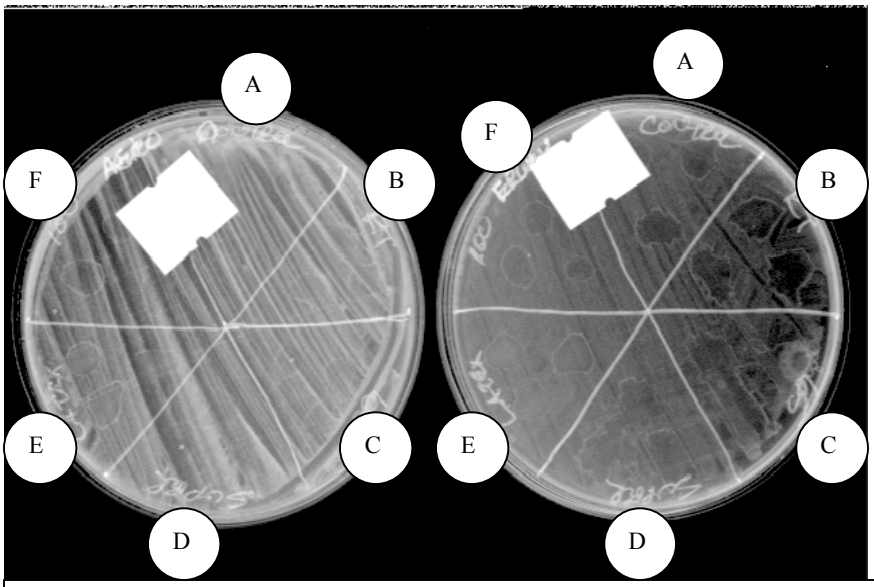
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D - Supernatant, E - Latex, F - 100 C

Figure 38 *Agrobacterium tumefaciens* antibiotic trials. Petri dishes containing *Agrobacterium tumefaciens* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments.



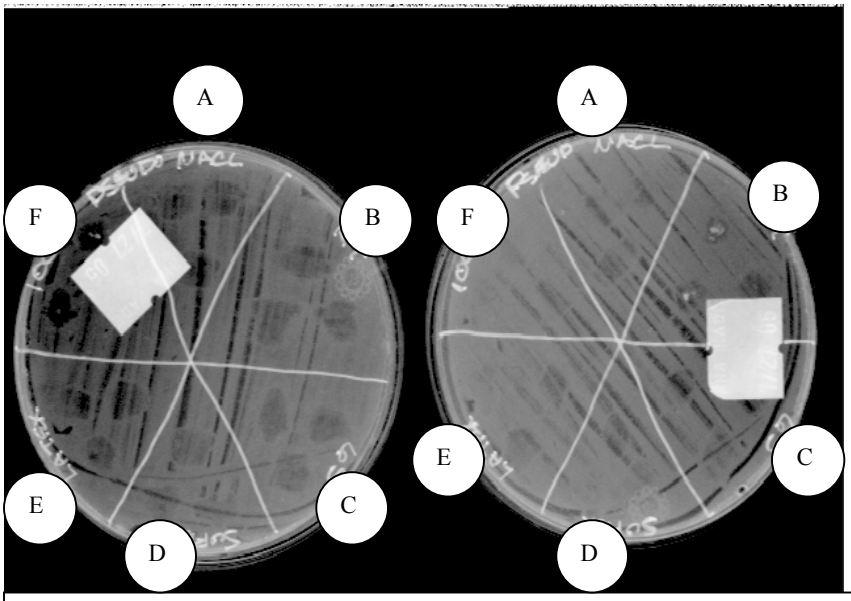
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D - Supernatant, E - Latex, F- 100 C

Figure 39 *Agrobacterium tumefaciens* repeat antibiotic trials. Repeat of *Agrobacterium tumefaciens* antibiotic trials to verify results of previous trials. Petri dishes containing *Agrobacterium tumefaciens* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments.



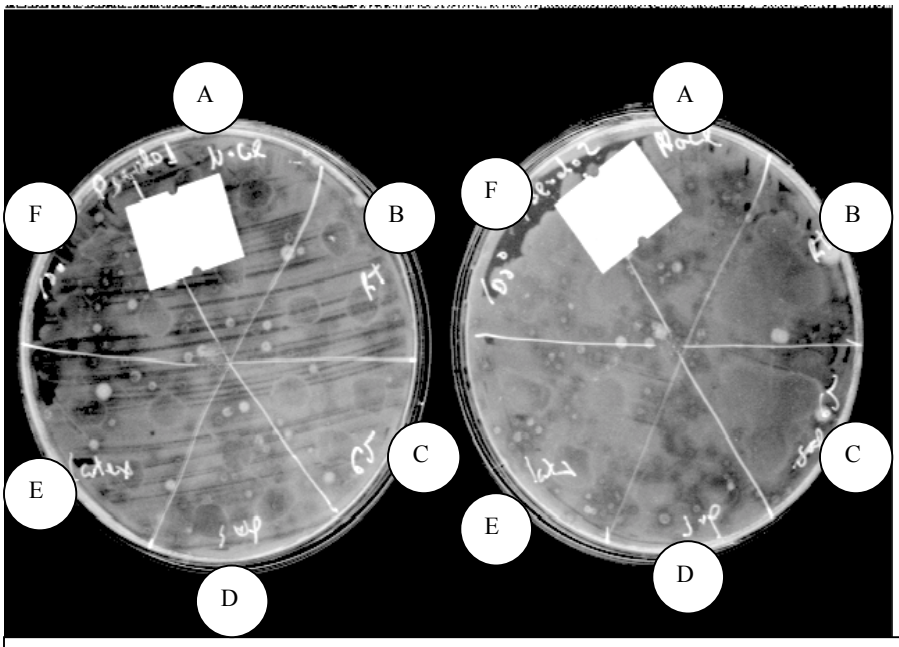
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D - Supernatant, E - Latex, F - 100 C

Figure 40 *Agrobacterium tumefaciens* third antibiotic trial. Third replicate of *Agrobacterium tumefaciens* antibiotic trials to verify results of previous trials. Petri dishes containing *Agrobacterium tumefaciens* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments.



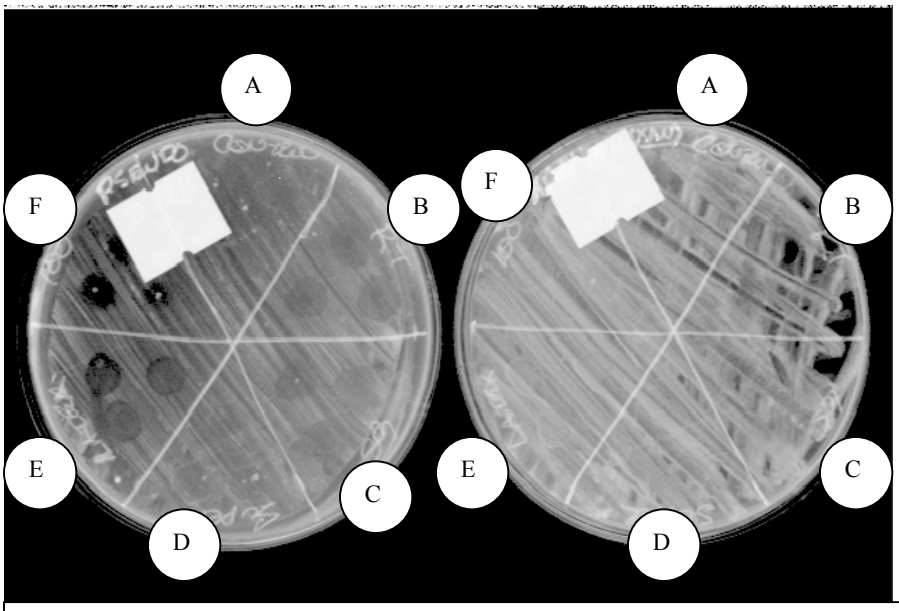
Treatments: A - Aqueous NaCl, B - Room temp. mix, C – 65 C, D – Supernatant, E – Latex, F - 100 C

Figure 41 *Pseudomonas syringae* antibiotic trials. Petri dishes containing *Pseudomonas syringae* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments.



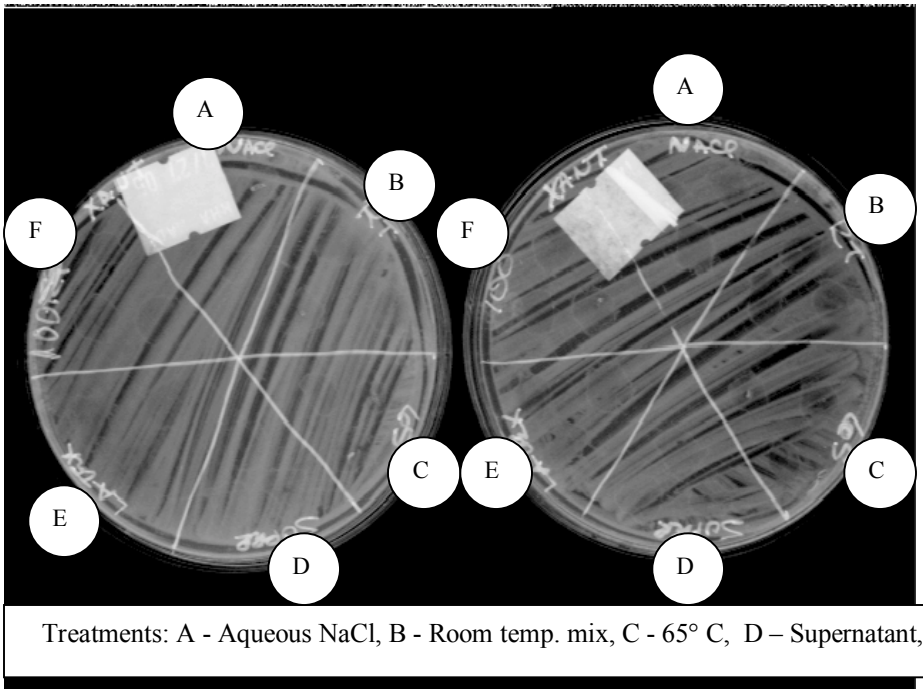
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D – Supernatant, E – Latex, F - 100 C

Figure 42 *Pseudomonas syringae* repeat antibiotic trials. Petri dishes containing *Pseudomonas syringae* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments. These plates had fungal contamination.



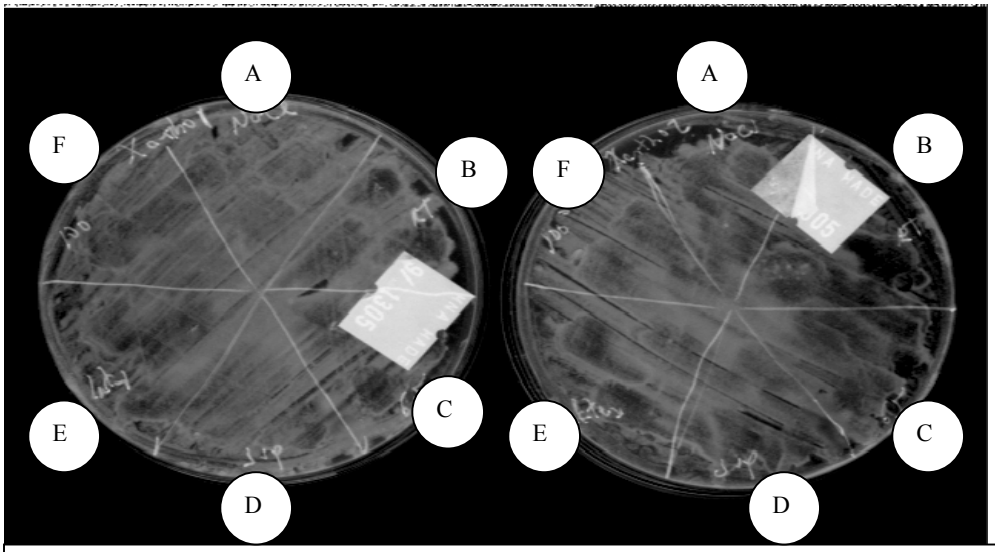
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D – Supernatant, E – Latex, F - 100 C

Figure 43 *Pseudomonas syringae and Xanthomonas sp.* repeat antibiotic trials. Petri dishes containing *Pseudomonas syringae and Xanthomonas sp.* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments.



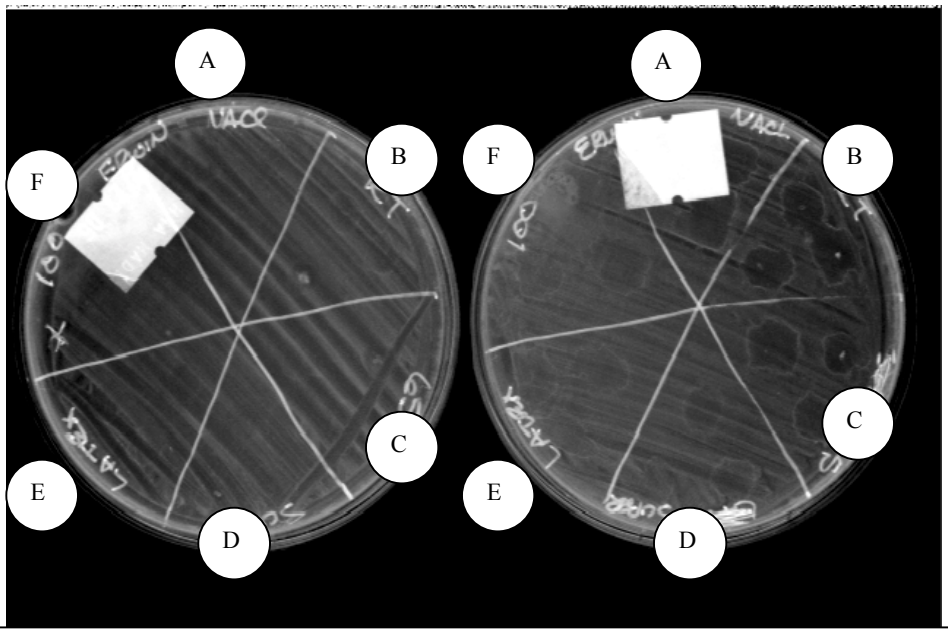
Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65° C, D – Supernatant, E – Latex, F - 100° C

Figure 44 *Xanthomonas sp.* antibiotic trials. Petri dishes containing *Xanthomonas species* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments.



Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D – Supernatant, E – Latex, F - 100 C

Figure 45 *Xanthomonas sp.* repeat antibiotic trials. Petri dishes containing *Xanthomonas species* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments.



Treatments: A - Aqueous NaCl, B - Room temp. mix, C - 65 C, D – Supernatant, E – Latex, F - 100 C

Figure 46 *Erwinia amylovora* antibiotic trials. Petri dishes containing *Erwinia amylovora* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments. Petri dish on right appears in the photo to have inhibition zones in all sectors but each zone supports bacterial growth with evidence of the margins of the sap application areas.

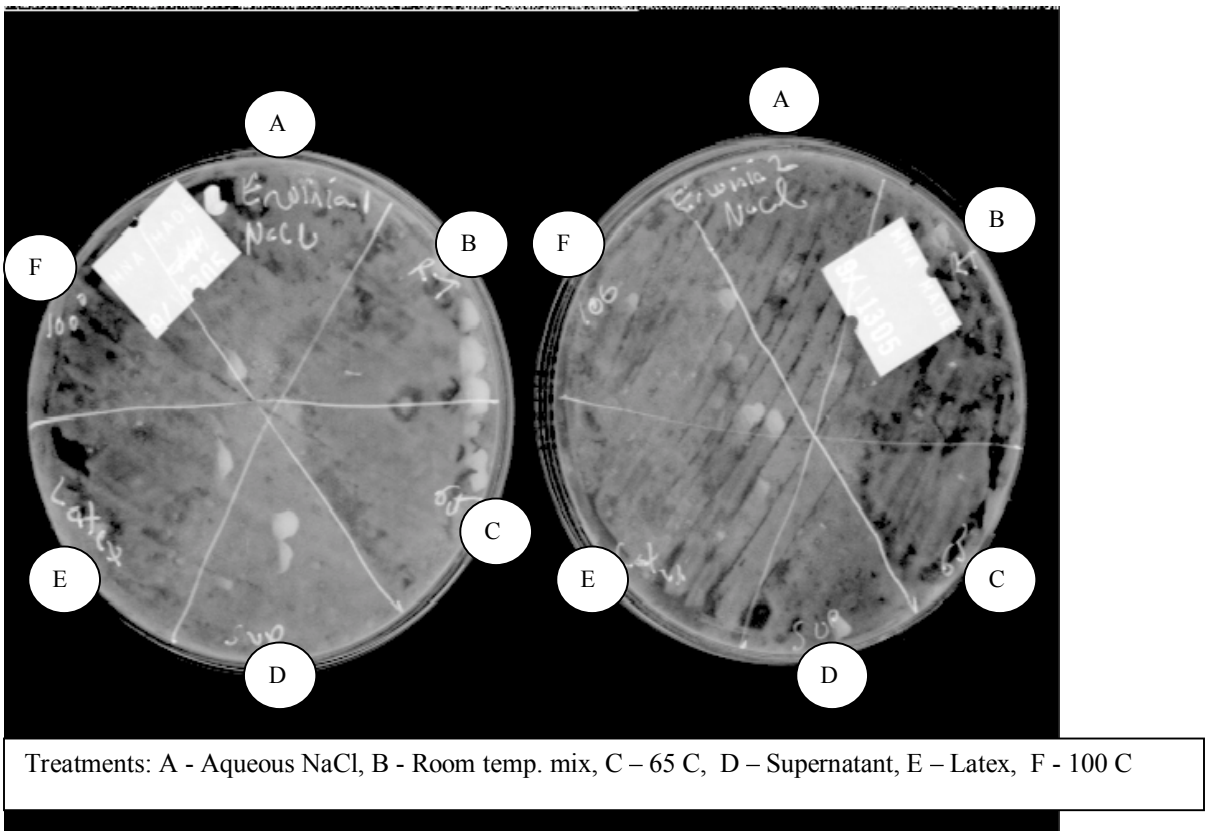


Figure 47 *Erwinia amylovora* repeat antibiotic trials. Repeat of *Erwinia amylovora* antibiotic trials to verify results of previous trials. Petri dishes containing *Erwinia amylovora* subjected to 6 different treatments. Three treatment drops were applied per sector. No inhibition zones were observed in any of the treatments.

Chemical Properties

The pure sap extract was too viscous for an accurate reading on an electronic pH meter. Therefore, latex sap was suspended in distilled water. This suspension had a pH of 5.8. A pH of 5.8 is classified as acidic and is approximately ten times more acidic than neutral pH.

Spectrophotometric analyses supported the visible observations that *Sciadopitys verticillata* sap was largely insoluble in water, but formed a suspension with small hydrophobic globules that over time settled to the bottom of the cuvette. As indicated by greater optical density, diluted latex sap subjected to vortexing existed as a suspension in water but dissolved in

ethanol (Table 21). The aqueous sap suspension was separated easily into latex and supernatant components by centrifugation. More latex sap stayed in suspension in water than in ethanol, as indicated by greater optical density of the water suspension and the supernatant (Table 21). Because the same amount of latex sap was put in equal amounts of each solvent, the lower optical density in ethanol indicates greater solubility.

Table 21 Spectrophotometer analysis data. Optical density at 420 nm.

Solvent	Suspension	Supernatant	Difference
Water	0.183	0.017	0.166
Ethanol	0.025	0.001	0.024
Total			0.142

CHAPTER 4

DISCUSSION

This study investigated three aspects of the biology of the unique conifer, *Sciadopitys verticillata*: methods to enhance success of asexual propagation; antimicrobial properties of the latex-like sap; and basic examinations of the physical and chemical characteristics of the latex-like sap (Table 1). Propagation trials were performed to develop a more efficient protocol for asexual propagation of *S. verticillata* by rooting stem cuttings. Reports of tree-to-tree variability in the ability to produce adventitious roots from stem cuttings was also investigated. Antimicrobial trials focused on inhibition of bacterial growth by the use of various suspensions containing latex sap. Eleven Gram-positive and Gram-negative bacteria were used in this study and included human pathogens/commensals, plant associated, and soil-borne bacteria (Table 2). Analysis was performed on *S. verticillata* sap to determine sap pH levels and to determine latex sap solubility in water and in ethanol. Sap pH was relevant to antimicrobial trials because bacteria used in this study prefer an environment within the 5.0 - 9.0 pH range. Latex solubility was relevant to the propagation trials because the latex may be forming an insoluble physical barrier which this current study hypothesized could be minimized by either physical or chemical treatments.

Propagation Trials

The propagation study examined the hypothesis that a water soak pre-treatment would prevent coagulation of the sap at the cut end of a stem without inhibiting the meristematic cells in *Sciadopitys verticillata* (Table 1). Significant improvement in rooting of stem cuttings over non-soaked controls was observed when water soak treatments were used (Tables10A, 10B, Figures 20, 21). Twenty-four hour water soaking treatments increased the probability of

adventitious root production for both June-collected new wood and March-collected old wood cuttings (Tables 10A, 10B, Figures 20, 21). Re-cutting the stems underwater provided a further gain in rate of rooting, but that increase was not significant compared to the air-cut water soak treatment (Tables 12A, 12B). Presumably, the water soak treatments reduced and/or removed latex, which can be observed to drain from the cut end of the stem into the soak water. Water soak treatments may thus reduce blocking of meristematic cells by coagulated latex. Re-cutting of stems may aid in exposing meristematic vascular cambium cells to air, to water, and to the rooting hormone that was applied to the cut ends. The advantage realized by cutting the stems underwater was relatively small and not significantly different from the water soak pretreatment so it is not likely to be useful in large-scale propagation operations unless a method that is less labor and time intensive can be developed.

Detergent treatment did not improve adventitious rooting of *S. verticillata* stem cuttings. Detergent-treated cuttings performed similarly to the control group in rooting success, but were less effective than the pure water treatments, indicating the addition of detergent reduced the beneficial effect of the water soak (Tables10A, 10B, Figures 20, 21). Detergent treatments, at the concentration used in this study, may not effectively dissolve and/or remove latex and may inhibit root growth. The 0.5% concentration used in these propagation trials is a relatively high concentration and may interfere with root formation by destroying cell wall or cell membrane integrity. Detergent is a polar molecule, with hydrophilic and hydrophobic regions that may react with the polar phospholipids of the cell membrane allowing cell contents to exit and/or allowing harmful agents to enter. Also, the detergent concentration used in this study may interfere with transport of water by clogging vascular tissues and/or by causing cells to become too flaccid or turgid due to either a hypotonic or hypertonic condition. Cell membrane damage may occur from

either condition if allowed to persist. Future studies may use lower detergent rates to determine if the decrease in rooting of stem cuttings observed in this study was due to concentration rates or unknown factors.

The control treatment produced roots at significantly lower proportions than cuttings in the water soak treatments (Tables 10A, 10B, Figures 20, 21). The difference in root production may be due to the accumulation of latex at the cut ends of stem cuttings, thereby acting as a physical block to new root development. This barrier may restrict cells' access to the rooting hormones, water, and air. Follow-up studies could determine if varying the soaking times of the stems in water increases the proportion of stem cuttings producing roots. For example, the 24 hours used in these trials may not be the optimal soaking time period to promote rooting or to break down any physical or chemical barrier. As little as 10 minutes may be sufficient to drain the latex from stems.

The data indicating that soaking was a more effective method of propagating from stem cuttings than the standard quick dip method may be used to develop propagation protocols for other difficult-to-root conifers. Although *Sciadopitys verticillata's* sap is insoluble in water, water soak treatments were effective because the latex-like sap streamed out of the submerged stems. Presumably, water tension aids in maintaining the flow of sap out of the stem. Many conifers contain non-latex resins, which may be inhibiting adventitious rooting in a manner comparable to latex sap in *S. verticillata*. Unless pine sap does not flow out in strands like the latex, a similar water soaking treatment could be used to prevent formation of a barrier formed by pine resins.

A goal of this study was to examine differences in the rooting ability of cuttings taken from different plants (Table 1). High tree-to-tree variability in rooting of stem cuttings was

reported by Waxman (1978), but no hypothesis for the cause of this variability was offered. Comparisons between trees used as sources of stem cuttings for the current study also indicated there were significant differences in rooting between source trees (Tables 9A, 9B, Figures 17, 18). The current study highlighted a likely cause for the apparent differences in rooting proportions among source trees because stem cuttings collected from trees growing in shaded environments rooted at significantly higher rates than cuttings from source trees in the sun. This difference was apparent only in old wood stem cuttings collected in March (Tables 9A, 9B, 17A, 18B, 19A, Figures 22, 23, 25). Seasonal effects and light environment effects are discussed separately but each show similar influences of shading that confounded the seasonal component.

One major conclusion of this study is the finding of a significant seasonal effect between new wood and old wood on rooting success. Old wood cuttings produced adventitious roots at higher proportions than new wood stem cuttings. Old wood stem cuttings were collected while the source tree was not producing expansion growth, and probably metabolically less active compared to warmer seasons. The seasonal effect may have been caused by a change in chemical or physical make-up of the sap (such as sap viscosity or levels of stored starch), levels of hormones, or there may have been a lower quantity of latex sap in the stems during the winter season. Because latex sap contains secondary metabolites that are products of photosynthesis-driven metabolic pathways, levels of latex sap are likely lower during the winter months, when sunlight is at its minimum (Lokvam et al. 2000). Waxman (1960) reported lower content during late winter compared to summer. A reduced level of latex was noted in the current study and may account for less mechanical obstruction of the meristematic cells needed for adventitious rooting of stem cuttings. Follow-up studies may be conducted to determine more precisely the optimal time of year for collecting stem cuttings from *S. verticillata* for propagation by performing

similar propagation trials on stem cuttings collected during each season or during each month of the year.

Another explanation for the significant seasonal effect on rooting may involve the levels of plant hormones known to either inhibit or promote adventitious rooting (such as auxins and gibberellins) that are likely to fluctuate during different seasons (Hu et al. 1999, Taiz and Zeiger 2002, Boerjan et al. 2003). For example, the plant hormone auxin is known to suppress side branch development while stimulating root development. The regeneration of vascular tissue is also controlled by auxin produced by the young buds directly above the wound site (Boerjan et al. 2003). Vascular differentiation is polar and occurs from leaves to roots (Taiz and Zeiger 2002). In woody perennials, auxin produced by growing buds in the spring stimulates activation of the cambium in a basipetal direction (Taiz and Zeiger 2002, Boerjan et al. 2003).

Chemical analysis of plant hormones during the different seasons was outside the scope of this study. Follow-up studies could include analysis of the latex-like sap and the vascular solutions during the different seasons and growth periods to determine if levels of hormones or nutrients fluctuate seasonally or stay relatively constant. Follow-up studies could also determine if the difference in rooting proportions was due to the quantity of sap in the stems and/or to the sap's chemical make-up. The amount and concentration of auxin applied to the cuttings could also be varied to determine an optimal level for successful rooting.

Different levels of cell lignification associated with old wood stem cuttings and cuttings from shaded trees, may also have an inhibitory effect on rooting because of cell expansion with division, level of cell maturity, and cell death of tracheary elements (Boerjan et al. 2003). This cell death means tracheary cells can no longer de-differentiate and divide due to lack of a nucleus and DNA (Ye and Droste 1996, Taiz and Zeiger 2002). One of the biochemical markers for cell

death of tracheary elements is degradation of nuclear DNA (Ye and Droste 1996, Soukup et al. 2004).

A second noteworthy conclusion of this study was the finding of a significant difference in the rooting ability between cuttings taken from trees growing in different light environments. Cuttings from shade-grown source trees rooted at higher rates than cuttings from sun-grown source trees taken at the same season. This effect of light environment was observed regardless of tree size, age, or the individual for both shade-grown tree cuttings (Table 17, Figures 23, 25, 26) and for sun-grown tree cuttings (Table 17, Figures 22, 24, 26). A re-occurring trend was noticed throughout this study that related the sun/shade environment to rooting proportions. An obvious difference between sun-exposed and shaded stems was that stems from shade locations were smaller in diameter, more flexible, and had less latex sap within the stem (Figure 13). The difference in stem diameter and flexibility between sun-exposed and shaded stems is likely due to the different degrees of maturity and lignification of stems.

The secondary xylem of trees and woody shrubs is composed of cellulose, lignin (phenolic polymer), and hemicelluloses at an approximate proportion of 2:1:1 (Hu et al. 1999). Cellulose microfibrils provide cell walls with tensile strength, while lignin encases the cellulose, providing rigidity to the cells (Hu et al. 1999). Lignin also aids in resistance to degradation by microbial attack (Whetten et al. 1998). Lignin deposition is one of the final stages of xylem cell differentiation and mainly takes place during secondary thickening of the cell wall (Donaldson 2001 cited in Boerjan et al. 2003). The secondary cell walls consist of three layers: the outer (S1), middle (S2), and inner (S3). Lignin deposition proceeds in different phases, each preceded by the deposition of carbohydrates, and starts at the cell corners in the region of the middle lamella and the primary wall (Donaldson 2001 cited in Boerjan et al. 2003). The bulk of lignin is

deposited after cellulose and hemicellulose have been deposited in the S3 layer (Baucher 1998 cited in Whetten et al. 1998, Saka 1985 cited in Boerjan et al., 2003, Donaldson 2001 cited in Boerjan et al. 2003). Secondary walls of vessels generally have higher lignin content than the secondary cell wall of fibers (Baucher et al. 1996 cited in Whetten et al. 1998, Donaldson 2001, Saka 1985 cited in Boerjan et al. 2003).

Cells in sun-exposed stems may be more mature than shade-exposed stems due to the increased levels of direct sunlight. Young growth contains a lower proportion of lignin and is more flexible than older growth (Constabel and Major 2005). As the plant cell matures more lignin is deposited to add strength, but flexibility is reduced (Constabel and Major 2005). Younger growth has higher water content and during deposition lignin may form chemical bonds with the hemicellulose component in the cell wall and gradually eliminates the water, forming a hydrophobic environment (Boerjan et al. 2003). The structure of lignins varies between plant species, between cell types within a single plant, and between different parts of the wall of a single cell. (Chen 1991 cited in Whetten et al. 1998, Soukup et al. 2004). In no case has a complete structure for any lignin been defined because it is a complex polymer (Chen 1991 cited in Whetten et al. 1998, Soukup et al. 2004).

Lignin content and composition have long been known to vary between the major groups of higher plants and between species (Freudenberg 1959 cited in Whetten et al. 1998, Driouch et al. 1996 cited in Whetten et al. 1998, Freudenberg 1965 cited in Whetten et al. 1998). The 2 major classes are, (i) gymnosperm lignins, which primarily contain guaiacyl subunits (G units) polymerized from coniferyl alcohol, and a small proportion of p-hydroxyphenyl units (H units) polymerized from p-coumaryl alcohol, and, (ii) angiosperm lignins which contain both syringyl units (S units), polymerized from sinapyl alcohol, and G units, with a small proportion of H

units. Exceptions to this basic classification are found in both gymnosperms and angiosperms (Sarkanen et al. 1971 cited in Whetten et al. 1998).

The phenotypic differences (levels of lignification) between sun- and shade-grown source trees observed in this study are consistent with results from earlier studies conducted on lignin proportions (Boudet et al. 1996 cited in Whetten et al. 1998, Campbell and Sederoff 1996 cited in Whetten et al. 1998, Monties 1989 cited in Whetten et al. 1998, Lewis and Yamamoto 1990 cited in Whetten et al. 1998). Variation in the proportion of these conventional lignin precursors is found within the cell wall, between cell types, and in response to biotic (pests and pathogens) and abiotic (mechanical) stresses (Monties 1989 cited in Whetten et al. 1998, Lewis and Yamamoto 1990 cited in Whetten et al. 1998, Boudet et al. 1996 cited in Whetten et al. 1998, Campbell and Sederoff 1996 cited in Whetten et al. 1998). The existence of more than one type of lignin and the precise regulation of monomer composition during development and stress responses strongly implies that alternative lignins may have different adaptive or functional properties (Monties 1989 cited in Whetten et al. 1998, Lewis and Yamamoto 1990 cited in Whetten et al. 1998, Boudet et al. 1996 cited in Whetten et al. 1998, Campbell and Sederoff 1996 cited in Whetten et al. 1998). The amount of lignin in a plant or given tissue can be maintained at a relatively constant level, even when an enzyme normally involved in the syntheses of lignin precursors is suppressed or impaired, and subunits not found in normal lignins are frequently present in such cases (Boudet et al. 1996 cited in Whetten et al. 1998, Ralph et al. 1997 cited in Whetten et al. 1998). However, the effect of reduced or altered lignin composition may not become obvious unless plants are tested under extreme conditions, such as drought, mechanical stress, or intense pathogen attack (Whetten et al. 1998).

Coniferin, the glucoside of coniferyl alcohol, accumulates to levels of a few percent of wet tissue weight in differentiated xylem of conifers (Terazawa et al. 1984 cited in Whetten et al. 1998, Savidge 1989 cited in Whetten et al. 1998). Hydrolysis of coniferin by a beta-glucosidase is considered essential before polymerization in the cell wall may proceed. A coniferin-beta-glucosidase (CBG) has been purified and characterized from pines (Leinhos et al. 1994 cited in Whetten et al. 1998, Dharmawardhana et al. 1995 cited in Whetten et al. 1998). CBG has been implicated in lignification by its enzymatic specificity, cellular location, and time of expression in development in both gymnosperms and angiosperms (Dharmawardhana et al. 1995 cited in Whetten et al. 1998).

Analysis of levels of lignification and rates of cell death may help explain seasonal differences in rooting of stem cuttings (Harkin and Obst 1973 cited in Whetten et al. 1998, Baucher et al. 1996 cited in Whetten et al. 1998, Boudet et al. 1996 cited in Whetten et al. 1998, Graber et al. 1996 cited in Whetten et al. 1998, Ralph et al. 1997 cited in Whetten et al. 1998, Sederoff and Chang 1991 cited in Whetten et al. 1998). Both laccases and peroxidase activities are associated with differentiation of xylem, although their activities on monolignols *in vitro* may differ. The most abundant activity is a laccase-like protein similar in physical properties and substrate specificity to previously characterized laccases purified from tree species (Sederoff and Chang 1991 cited in Whetten et al. 1998, Ralph et al. 1997 cited in Whetten et al. 1998). Studies by Harkin and Obst (1973 cited in Whetten et al. 1998) detected laccase activity in green ash by sampling at a biologically relevant time of year (from growing trees in late spring, rather than dormant trees in early spring) and using a different substrate. After fulfilling cellular activities necessary for building up a secondary wall, developing tracheary elements undergo cell death to remove their cellular contents, and in the case of vessel elements, their ends are perforated to

form tubular columns called vessels (Ye and Droste 1996). This release of cellular contents includes the removal of the cell's DNA and therefore eliminates the cell's ability to de-differentiate into adventitious roots.

This study found no significant differences in rooting between stem cuttings from different aged plants (Tables 7A, 7B, Figures 15, 16). The proportion of stem cuttings that produced roots was not related to cutting source tree, tree height, or tree age, which indicates that cuttings from trees of any age have similar abilities to produce roots. These similarities were not unexpected because stem cuttings collected from any tree regardless of age, developmental stage, or genetic diversity should have nucleated cells that contain all the genetic material (DNA) needed for cell totipotency, cell differentiation, and/or de-differentiation into adventitious roots. Similarly, all stem cuttings collected from any tree, regardless of age, developmental stage, or genetic background should have meristematic cells capable of dividing and developing adventitious roots.

Cuttings from sun-grown trees tended to maintain viability without producing roots and they were often heavily callused. Callus consist of de-differentiated cells composed primarily of callose, which is a β-1,3-glucan that is synthesized by an enzyme in the plasma membrane and is deposited between the plasma membrane and the cell wall (Taiz and Zeiger 2002, Nishikawa et al. 2005). Callose is synthesized in functioning sieve elements in response to damage and other stresses, such as mechanical stimulation, high temperatures, and in preparation for dormancy (Taiz and Zeiger 2002, Nishikawa et al. 2005). The deposition of wound callose disappears from the pores once the sieve elements have recovered from the damage (Taiz and Zeiger 2002, Nishikawa et al. 2005). With enough time and the right environmental conditions, the callused stem cuttings may eventually produce roots. A revised hypothesis, postulating increased root

production with increased time under the right conditions, may be tested by extending the time period cuttings are exposed to controlled greenhouse conditions (heat, humidity, and intermittent water mist) to longer than the 6 months used in the current study. Rooting hormones could also be reapplied to stem cuttings with intact callus and cuttings with callus physically removed, to determine if there is an advantage to hormone re-treatment in addition to increased rooting time.

While not a component of the current study, the observation of greater amounts of sap in the cuttings from sunny sites and may have helped to prevent the cuttings from dying of rot in wet conditions. It became evident that for optimal rooting rates, the greenhouse environmental conditions of the cuttings must be adjusted depending on the type of cutting used. Most cuttings from shade source trees rotted if they did not produce roots. The cuttings from source trees growing in the sun, with more waterproof lignin, can survive more water and higher proportions of peat moss in the media without rotting below the soil line. The cuttings from source trees growing in shade may need less water and higher proportions of perilite in the media to ensure proper drainage and prevent rot. Many of the dead shade cuttings showed evidence of rot below the soil surface. This could also be an indication of the possible connection with the degree of cell lignification and successful rooting of *Sciadopitys verticillata.* Lignin aids in waterproofing the secondary cell wall and its production is influenced by environmental conditions as nutrient availability, mechanical damage, and exposure to sunlight (Dandekar et al. 1993). Future studies may determine the optimal water levels to maintain around the cuttings. Future propagation studies may vary both the amount of water applied to the cuttings and/or may vary soil drainage characteristics by adjusting the proportions of peat moss and perilite. Increasing proportions of perilite will increase water drainage and may aid in prevention of stem cutting rot.

Antibiotic Trials

One of the goals of this study was to assess antibacterial properties of *Sciadopitys verticillata* sap against a diverse panel of bacterial species to test the hypothesis that there would be no antimicrobial activity attributable to the latex-like sap of *S. verticillata* (Table 1). Eleven species of bacteria were subjected to a battery of sap treatments (Table 3). Six of these species of bacteria showed inhibition (Table 20, Figures 29, 30, 31, 32, 33, 34, 35, 36). Therefore, *S. verticillata* sap has antimicrobial properties against some species of bacteria but displayed no observable antimicrobial effect on other bacterial species (Table 20, Figures 37, 38, 39, 40, 41, 42, 43). Previous studies conducted on latex from *Asclepias syriaca* indicated no antimicrobial activity associated with either whole or fractioned latex when tested against *Bacillus subtilis, Clostridium sporogenes, Escherichia coli, Staphylococcus aureus*, or *Rhodospirillum rubrum* (McCay and Mahlberg, 1973). Although no antibiotic activity against *Escherichia coli* was detected in the current study, antibiotic activity was detected against *Bacillus subtilis* and *Staphylococcus aureus* (Table 20, Figures 29, 31, 32, 36, 37). Future studies may determine the active ingredient(s) associated with the antimicrobial effect discovered in this study by performing gas chromatographic (GC) analysis of the latex of *S. verticillata*. Comparison of GC profiles to profiles of known antibiotic agents may detect latex components to be further explored as the potential source of the antimicrobial activity.

Aqueous sodium chloride (NaCl) solution and a pencil subjected to the stem cutting sterilization procedures were used as controls in this study. The absence of inhibition zones on both sets of controls demonstrates that the latex-like sap was the source of the antibiotic activity (Table 20, Figures 29, 30, 31, 32, 33, 34, 35, 36,). A further indicator of the antibacterial activity

of the sap was that only some species were inhibited (Table 20, Figures 29, 30, 31, 32, 33, 34, 35, 36,).

Some Gram-positive and Gram-negative bacteria were inhibited by the sap treatments, however some strains of Gram-negative bacteria were not inhibited (Table 20, 37, 38, 39, 40, 41, 42, 43, 44). This indicates that the pattern of inhibition is probably not related to ability of a bacteria to react with Gram stain and therefore suggests the mechanism of inhibition is not related to characteristics distinguishing Gram-negative and Gram-positive bacterial cell walls.

The largely insoluble sap suspension was easily separated into latex-enriched and supernatant components by centrifugation. The absence of inhibition zones on the bacteria treated with supernatant indicates the supernatant was not the source of the antibiotic activity (Table 20). The antibiotic activity was clearly associated with the latex sap.

Antibiotic activity was not lost following heating of the mixture to 65-85 C or to 100 C, which indicates the antibacterial active component is not denatured by heat (Table 20). Maintenance of antibiotic activity after heat treatments suggests the active ingredient is not a protein. Moreover, the strength of the antibiotic activity was demonstrated by retention of activity against *Staphylococcus epidermidis* and *Neisseria cinerea* following 100:1 dilution of the initial sap suspension (Table 20, Figures 33, 34). The initial sap suspension was an approximate 1:12 dilution of sap. Thus, an approximate 1:1200 of sap maintained antibiotic activity. Similar studies using bioactive plant extracts on methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* have been conducted using extracts from bark, leaves, fruits, bulbs, and flowers from plants unrelated to *Sciadopitys* (Aqil et al. 2005, Moulari et al. 2006). The current study attained similar results using *Sciadopitys verticillata* latex sap;

however, the current study detected antibiotic effects against a wider range of bacteria species, some of which lack antibiotic resistances.

No relationship between bacterial ecological niche and inhibition was evident, but none of the 4 plant pathogens were inhibited. However, only a small sample of potential plant pathogens were tested and the possibility of inhibiting other plant pathogens cannot be ruled out. Because *Sciadopitys verticillata* is not susceptible to infection by the pathogens tested, *Sciadopitys* may not have evolved resistance to these bacteria.

Chemical Properties

A sap suspension pH of 5.8 indicates the cause of bacterial inhibition detected in this study was not due to an alteration of the pH environment beyond expected tolerances of bacteria under study. *Sciadopitys verticillata* sap is neither too acidic nor too basic to deter bacterial growth.

A goal of this study was to determine if water or ethanol was the better solvent for the latex sap of *S. verticillata.* Spectrophotometric analysis of latex sap solutions that measured light absorbance and optical densities at 420 nm indicated *Sciadopitys* latex formed a suspension in water but it solubilized in ethanol (Table 21). In water, latex can be easily separated from the solvents by centrifugation. Latex sap came out of solution relatively quickly and settled to the bottom of the container, in a manner similar to sand separating from water and settling to the bottom in a suspension of coarse soil. In contrast, in ethanol the solution cleared upon addition of sap as would a solution of salt in water.

Conclusions

The most effective protocol for the asexual propagation of *Sciadopitys verticillata* by rooting of stem cuttings is to collect stem cuttings from trees growing in a shaded environment during the early spring when the tree is not undergoing extension growth. Stem cuttings from shade-grown trees have a tendency to be smaller in diameter and contain less latex sap. The deposition of latex sap at the cut end of stem propagules may act as a rooting barrier. This presumed barrier can be overcome by soaking freshly cut stem cuttings in water for 24 hours before applying hormones and placing cuttings in soil media, under mist, with 50% shade for approximately 6 months.

This study showed that no single factor explained the differences in rooting proportions of *Sciadopitys verticillata* stem cuttings. Plants are complex organisms with great phenotypic and genotypic variation between and within species. This complexity adds to our current lack of understanding of many of the interrelated hormonal and metabolic pathways and interactions. Hormone levels, metabolism, light environment of source tree, and lignification are all important to cell growth, development, differentiation, and de-differentiation that may be required for adventitious root production. Latex sap most likely forms a physical barrier to rooting because levels of cell lignification were not altered by water soak treatments yet rooting proportions increased in response to water soaking. This increase may be attributed to the removal of latex sap and the apparent reduction of the hypothesized barrier to adventitious root formation.

Latex sap of *Sciadopitys verticillata* showed antimicrobial activity against certain bacterial species. Further investigation into the antimicrobial properties of *Sciadopitys verticillata's* sap to include tests of viruses, fungi, and bacteria with known resistances to antibiotics can be conducted in the future. Bacterial inhibition attributed to latex pH has been

ruled out by this study. The substance in the latex sap that is causing the antibiotic effect remains unknown, as does the mechanism of inhibition. Future studies could focus on identifying the source and mechanism of the antibiotic activity. The sap constituents could be compared to known antibiotics to provide insight into whether the molecular structure is novel enough to explore its use as a source for potential clinical use.

Future studies of the chemical and physical properties of the latex sap of *Sciadopitys verticillata* may include separating the latex into its components to detect the source of antimicrobial activity detected in this study. Gas chromatography could also aid in defining constituents of the sap. Future solubility studies could be conducted using additional solvents such as benzene, phenol, dimethyl sulfoxide, and acetone.

In closing, *Sciadopitys verticillata* is a rare and unusual ornamental tree difficult to asexually propagate and it is a potential source for new antibiotics. *Sciadopitys verticillata* is increasing in popularity with home gardeners, is still difficult to find, and is a relatively expensive ornamental plant. The results of this study may make it an intriguing model for the study of latex and its impact on adventitious rooting and a potential investment for the future of ornamental horticulture. Thomas Jefferson wrote about his services to his country and stated, “The greatest service which can be rendered any country is to add a useful plant to its culture" (Jefferson, 1800). *Sciadopitys verticillata* may be just such a plant.

REFERENCES

Aqil, A., A. Sajjad, A. Khan, M. Owais, I. Ahmad. 2005. Effect of certain bioactive extracts on clinical isolates of β-lactamase producing methicillin resistant *Staphylococcus aureus.* J. Basic Microbiol. 45(2): 106-114.

Axsmith, B.J., E.L. Taylor, T.N. Taylor. 1998. The limitations of molecular systematics: a palaeobotanical perspective. Taxon 47: 105-108.

Blazich, F.A., R.D. Wright. 1986. Growth response of umbrella pine as influenced by temperature, photoperiod, and chilling. J. Environ. Horticulture 4: 145-148.

Boerjan, W.J., J. Ralph, M. Baucher. 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54: 519-546.

Bravo, L.M., J. Hermosilla, C.E. Salas. 1994. A biochemical comparison between latex from *Carica candamarcensis* and *Carica papaya*. Brazilian J. Med. Biol. Res. 27: 2831-2842.

Calvin M. 1979. Petroleum plantations for fuel and materials. BioScience 29: 533-538.

Calvin M. 1984. Renewable fuels for the future. Journal of Applied Biochemistry 6: 3-18.

Constabel, P.C., I.T. Major. 2005. Molecular biology and biochemistry of induced insect defense in *Populus*. Chapter 5 in, "Recent Advances in Phytochemistry", J.T. Romeo, ed. Elsevier Press, New York, pp. 119-143.

Dallimore, W., A.B. Jackson, S.G. Harrison. 1967. A Handbook of Coniferae and Ginkgoaceae, 4th ed. St. Martin's Press, New York, p. 729.

Dandekar, A.M., G.H. McGranahan, D.J. James. 1993. Transgenic woody plants. Chapter 7 in Transgenic Plants vol. 2, S-D. Kung and R. Wu, eds. Academic Press, pp 129-151.

Dirr, M.A. 1990. Manual of Woody Landscape Plants: Their Identification, Ornamental Characteristics, Culture, Propagation, and Uses. Stipes Publishing, Champaign, IL. p. 785.

Dorman, H.D., S.G. Deans. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88: 308-312.

Douds, D.D. Jr., G. Becard, P.E. Pfeffer, L.W. Doner, T.J. Dymant, W.M. Kayser. 1995. Effect of vesicular-arbuscular mycorrhizal fungi on rooting of *Sciadopitys verticillata* Sieb. & Zucc. cuttings. HortScience 30: 133-134.

Farjon, A.S., G.S. Ortiz. 2003. Cone and ovule development in *Cunninghamia* and *Taiwania* (Cupressaceae sensu lato) and its significance for conifer evolution. American Journal of Botany 90: 8-16.

Francheschi, V.R., T. Hrekling, A.A. Berryman, E. Christiansen. 1998. Specialized phloem parenchyma cells in Norway spruce (Pinaceae) bark are an important site of defense reactions. American Journal of Botany 85(5): 601-615.

Flemer, W. III. 1961. Further experiences in rooting *Sciadopitys verticillata* cuttings. Combined Proc. Intl. Plant Propagator's Soc. 11: 104-106.

Goodhart, F.W. 2000. Propagation aspects of *Sciadopitys verticillata* and *Sequoiadendron giganteum* 'Hazel Smith'. Combined Proc. Intl. Plant Propagator's Soc. 50: 222-226.

Halladin, P. 1991. *Sciadopitys verticillata.* American Nurseryman 8: 73-74.

Hu, W.J., S.A. Harding, J. Ling, J.L. Popko, J. Ralph, D.D. Stokke. C.J. Tsai, V.L. Chiang. 1999. Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. Nature Biotech.17: 808-812.

Hubner, A.T. 2003. Treatment with *Hypercium perforatum* L. does not trigger decreased resistance in *Staphylococcus aureus* against antibiotics and Hyperforin. Inter. Journal of Phytotherapy and Phytopharmacology 3: 206-209.

Jefferson, T., 1800. The Writings of Thomas Jefferson. Literary Classics of the United States, Inc., New York, NY, p. 702-704.

Langenheim, J.H. 2003 Plant Resins: Chemistry, Evolution, Ecology, and Ethnobotany. Timber Press, Portland, OR. pp. 23-66, 203-205, 452-455.

Liggieri, C., M.C. Arribere, S.A. Trejo, F. Canals, F.X. Aviles, N.S. Priolo. 2004. Purification and biochemical characterization of asclepain c I from the latex of *Asclepias curassavica*. Protein Journal 23: 403-411.

Lin, R.D., Y.P. Chin, M.H. Lee. 2005. Antimicrobial activity of antibiotics in combination with natural flavonoids against clinical extended spectrum β-lactamase producing *Klebsiella pneumoniae*. Phytotherapy Research 19: 612-617.

Lindberg, L.E., S.M. Willfor, B.R. Holbom. 2004. Antibacterial effects of knotwood extractives on paper mill bacteria. Journal for Industrial Microbiology and Biotechnology 31: 137-147.

Lokvam, J., J.F. Braddock, P.B. Reichardt, T.P. Clausen. 2000. Two polyisoprenylated benzophenones from the trunk latex of *Clusia grandiflora* (Clusiaceae). Phytochemistry 55: 29-34.

Madigan, M.T., J.M. Martinko. 2006. Brock Biology of Microorganisms. 2nd ed. Pearson Prentice Hall, Upper Saddle River, NJ, pp 157-159.

McCay, S., P. Mahlberg. 1973. Study of antibacterial activity and bacteriology of latex from *Asclepias syriaca*. Antimicrobial Agents and Chemotherapy 3: 247-253.

Miller, C.N. 1999. Implications of fossil conifers for the phylogenetic relationships of living families. Botanical Review 65: 239-277.

Moulari, B., Y. Pellequer, H. Lboutounne, C. Girard, J.P. Chaumont, J. Millet, F. Muyard. 2006. Isolation and in vitro antibacterial activity of astilbin, the bioreactive flavanone from the leaves of *Harungana madagascariensis*. Journal of Ethnopharmacology 8: 4126-4133.

Nishikawa, S., G.M. Zinkl, R.J. Swanson, D. Maruyama, D. Preuss. 2005. Callose (β-1,3 glucan) is essential for *Arabidopsis* pollen wall patterning, but not tube growth. Plant Biology 5: 22-29.

Phillipe, M., H. Meon, G. Lambert, B. Erdei, F. Thevenard, B. Gomez. 2002. A palm-tree and *Sciadopitys* swamp-forest from the Neogene of Bresse (eastern France). Systematic Paleontology 1: 221-225.

Sakagami, Y., M. Iinuma, K.G. Piyasena, H.R. Dharmaratne. 2005. Antibacterial activity of alpha-mangostin against vancomycin-resistant *Enterococci* and synergism with antibiotics. Inter. Journal of Phytotherapy and Phytopharmacology 12: 203-210.

Schlarbaum, S.E., T. Tsuchiya. 1985. Karyological derivation of *Sciadopitys verticillata* Sieb. et Zucc. from a pro-taxodiaceous ancestor. Botanical Gazette 146: 264-77.

Soukup, A., J. Mala, M. Hrubcove, J. Kalal, O. Votrubova, M. Cvikrova. 2004. Differences in anatomical structure and lignin content of roots of pedunculate oak and wild cherry-tree plantlets during acclimation. Biologia Plantarum 48: 481-489.

Stefanovic, S., M. Jager, J. Deutsch. 1998. Phylogenetic relationships of conifers inferred from partial 28S rRNA gene sequences. American Journal of Botany 85: 688-97.

Taiz, L., E. Zeiger. 2002. Plant Physiology. 3rd ed. Sinauer Associates, Sunderland, MA. pp 2-51, 196-197, 293-303, 349-361, 424-447.

Takaso, T., P.B. Tomlinson. 1991. Cone and ovule development in *Sciadopitys* (Taxodiaceae-Coniferales). American Journal of Botany 78: 417-28.

Waxman, S. 1957. Effects of daylength on the germination of *Sciadopitys verticillata*. Combined Proc. Intl. Plant Propagator's Soc. 7: 71-72.

Waxman, S. 1960. Propagation of *Sciadopitys verticillata*. Combined Proc. Intl. Plant Propagator's Soc. 10: 178-181.

Waxman, S. 1978. Propagation of umbrella pine (*Sciadopitys verticillata*)-clonal differences in root initiation. Combined Proc. Intl. Plant Propagator's Soc. 28: 546-550.

Whetten, R.W., J.J. MacKay, R.R. Sederoff. 1998. Recent advances in understanding lignin biosynthesis. Ann. Rev. Plant Physiol. Plant Molec. Biol. 49: 585-609.

Wolff, R.L., L.G. Deluc, A.M. Marpeau. 1996. Conifer seeds: oil content and fatty acid composition. J. Am. Oil Chem. Soc. 73: 765-771.

Wollenweber, E., L. Kraut, R. Mues. 1998. External accumulation of biflavonoids on gymnosperm leaves. Z. Naturforsch 53: 946-950.

Yamamoto, S. 1988. Seedling recruitment of C*hamaecyparis obtusa* and *Sciadopitys verticillata* in different microenvironments in an old-growth *Sciadopitys verticillata* forest. Botanical Magazine of Tokyo 101(1061): 61-71.

Ye, Z.H., D.L. Droste. 1996. Isolation and characterization of cDNAs encoding xylogenesis-associated and wounding-induced ribonucleases in *Zinnia elegans*. Plant Mol. Biol. 30: 697-709.

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